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14. ABSTRACT

Because it is now apparent that a significant proportion of patients chronically treated with imatinib develop resistance due to the acquisition of mutations in the kinase domain of BCR-ABL, our aim was to generate a potent inhibitor of BCR-ABL by targeting regions outside the ATP binding site of this enzyme, as these compounds offer the potential to be unaffected by mutations that make CML cells resistant to imatinib. Screening of a novel library of small molecule kinase inhibitors which are unrelated to ATP or other purine and pyrimidine nucleosides using the high through-put assay led to the identification of three six new compounds series. Of these, three compounds were found to be most active against all of the imatinib-resistant forms of BCR-ABL including the T315I mutation in vitro and in vivo assays. In addition, these compounds were also found to inhibit the proliferation of and induce apoptosis of leukemic cell lines that express the V617F mutant form of JAK2 and establish their utility for the treatment of myeloproliferative disorders arising due to mutations in JAK2. We provide a detailed description of their biological activity and mechanism action of these compounds.

15. SUBJECT TERMS
BCR-ABL, Imatinib Resistance

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Introduction

Imatinib, which is an inhibitor of the BCR-ABL tyrosine kinase, has been a remarkable success for the treatment of Philadelphia chromosome positive CMLs (Deininger et al, 2005). However, a significant proportion of patients chronically treated with imatinib develop resistance due to acquisition of mutations in the kinase domain of BCR-ABL. Mutations occur at residues directly implicated in imatinib binding or, more commonly, at residues important for the ability of the kinase to adopt the specific closed (inactive) conformation to which imatinib binds (Deininger et al, 2005). Because of the frequency of mutations, efforts are now focused on the identification of novel inhibitors that are active against imatinib resistant mutants of BCR-ABL.

In response to this demand, other classes of promising compounds have recently emerged. One compound, AP23464 (O'Hare et al, 2004), is a trisubstituted purine and inhibits wild-type BCR-ABL and Src family kinases at nonomolar concentrations. While this agent is also inhibitory against many imatinib-resistant forms of BCR-ABL, it was found to be ineffective against BCR-ABL^{T315I}, the mutation that is present in the greatest percentage of patients who have developed imatinib resistance. Another compound, BMS-354825 (Shah et al, 2004), is an orally bioavailable inhibitor of BCR-ABL and Src kinases and is inhibitory to nearly all imatinib-resistant forms of BCR-ABL. It too is unable to overcome imatinib resistance caused by T315I. Finally, a third compound, termed AMN107 (Weisberg et al, 2005) is closely related to imatinib and has a greater affinity (approximately 20-fold) for wild-type BCR-ABL (reviewed in O'Hare et al, 2005). As a result, most of the imatinib-resistant forms of BCR-ABL are also 20-fold more sensitive to this compound, with the exception of T315I, which in insensitive to AMN107 (Weisberg et al, 2005). As this particular mutation is the most resistant to imatinib and emerges in the largest percentage of patients who develop resistance (reviewed in Deininger et al, 2005), there is an urgency to develop alternative compounds that are capable of inhibiting this particular (as well as other) amino acid substitution.

BODY

Work Accomplished by Dr. Reddy's Group at the Fels Institute

Because it is now apparent that a significant proportion of patients chronically treated with imatinib develop resistance due to the acquisition of mutations in the kinase domain of BCR-ABL, our aim was to generate a potent inhibitor of BCR-ABL by targeting regions outside the ATP binding site of this enzyme as these compounds offer the potential to be unaffected by mutations that make CML cells resistant to imatinib. Studies with kinase inhibitors have identified three general mechanisms for pharmacological inhibition of kinase activity: (1) Direct binding in the ATP binding site, (2) binding in the substrate-binding site, and (3) engagement of an allosteric site which results in the altered conformation of the kinase resulting in a block to proper substrate phosphorylation.

One of the important facts that has emerged in the past one year is the realization that non-ATP competitive inhibitors often show little or no kinase inhibitory activity in *in vitro* assays (Adrian et al, 2006). An explanation for this lack of *in vitro* activity could be the absence of kinase-associated proteins in *in vitro* reactions which appear to dictate the specificity of kinase reactions *in vivo*. This explanation appears to be supported by the fact that many of the kinases phosphorylate artifical substrates such as casein, IgG and synthetic peptide substrates, which are not normally the targets for these enzymes. In addition, the *in vitro* kinase reactions are usually carried out in the presence of an excess amount of substrate which does not favor inhibition by substrate-competitive inhibitors.
In order to more efficiently screen our library of compounds for BCR-ABL inhibitors in a cell-based assay, we set up a high thru put screen employing a 96 well format in combination with spot blot hybridization using infrared technology developed by Li-Cor, Biosciences, NE. The power of this screen lies in the increased sensitivity, low background-to-noise ratio and the ability to use two color detection with little bleed-

through between detection channels. This high thru put assay for cell-based enzyme inhibition was found to be very sensitive for non-ATP competitive inhibitors. Briefly, we plated 2.5 x 10^4 K562 cells per well in a 96 well plate. The cells were treated in duplicate for 2 hours with 10 uM concentration of each compound or 10 uM of imatinib, which was used as a positive control. DMSO alone was used as the vehicle control. Following the incubation, the plate was centrifuged for 20 minutes to pellet the cells. The cell pellets were then lysed in the plate and the cellular debris was removed by high speed centrifugation. The cell lysates were then spotted onto nitrocellulose (Millipore) and the membrane was processed for hybridization according to the manufacture's instructions. The blot is hybridized using two primary antibodies simultaneously. One antibody is specific for phospho-bcr-abl (Cell Signaling Rabbit polyclonal:CS-390) and the second is specific for Actin (Sigma monoclonal:A2228, loading control). The blot was then hybridized with two secondary antibodies that are specific for either murine or rabbit IgG molecules. These antibodies were conjugated with infrared dyes that excite at either 685 or 785 nm excitation wavelengths. The blots were scanned using two diode lasers (Odyssey scanner) where the detection is based on filtration by two independent detection channels. The image was then stored digitally and analyzed by many different parameters, including direct quantification using software provided by the manufacturer. Compounds that inhibit BCR-ABL kinase activity were identified by their ability to inhibit the autophosphorylation of BCR-ABL (as measured by reduction in the 700 nm fluorescence signal), and were compared to the reduction caused by imatinib, which was used as a positive control. This assay allowed us to screen up to 44 compounds in duplicate at a time using very limited number of man-power hours.

Figure 1 is an example of a typical 96 well assay screen. We treated K562 cells with 44 new compounds from our compound library, at a final concentration of 10 uM in duplicate. The top panels show images derived from each independent channel. Panel A shows the detection of phosphorlated form of BCR-ABL. Duplicate wells located in positions 12:CD were completely negative for 700 nm signal but had normal levels of total protein as determined by the 700 nm signal shown in panel B. Panel C shows the dual color image when the red and green channels are super imposed upon one another.

Here one can clearly see one positive compound. The final scan identified one compound in position 12:CD that had the ability to completely inhibit the kinase activity of BCR-ABL. The amount of inhibition was equal to or better than that found for 10 uM of imatinib, located in position 1:FG.

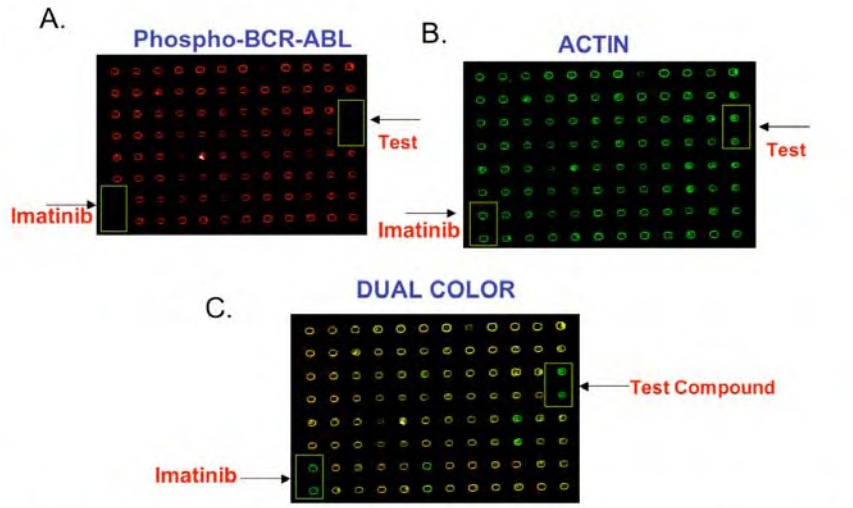

Fig. 1. Dual Infrared Based High Thru Put Screen. K562 cells were plated in a 96 well plate and treated with 10 uM of each test compound or imatinib for 2 hours. The plate were centrifuged, cells were lysed and total cell lysate was spotted onto a nitrocellulose filter paper. The blot was hybridized simultaneously with anti phosphospecific BCR-ABL (red:polyclonal) and anti actin (green:monoclonal) antibodies. The blot was then treated with secondary antibodies conjugated with infrared dyes and the final blot was scanned and imaged using the Odyssey scanner (Li-Cor).

Following the identification of inhibitors of BCR-ABL, we next performed secondary assays to reproduce the primary screen and to more closely determine the IC_{50} value of the compound. This was performed by treating K562 cells with increasing concentrations of the compound (dose response) and then performing a typical western blot assay using the infrared based screening protocol described for western blotting.

Screening of a novel library of small molecule kinase inhibitors which are unrelated to ATP or other purine and pyrimidine nucleosides (provided to us by Onconova Therapeutics) using the high thru put assay described above in combination with kinase assays led to the identification of six new compound series which include the ON27 series (represented by ON271300), the ON0 series (represented by ON015290, ON044580 and ON045000) the ON88320 series and a new series of compounds represented by ON96030. Of these, ON044580 and ON045000 were found to be most active against all of the imatinib-resistant forms of BCR-ABL including the T315I mutation. In addition, ON044580, ON045000 and ON96030 were found to be dual inhibitors of BCR-ABL and JAK-2, which makes them ideal agents for the treatment of other myeloproliferative diseases in addition to CML. We therefore focused our first

years effort on the pre-clinical development and delineation of mechanism of action of these three compounds.

Inhibition of *in vivo* kinase activity of BCR-ABL

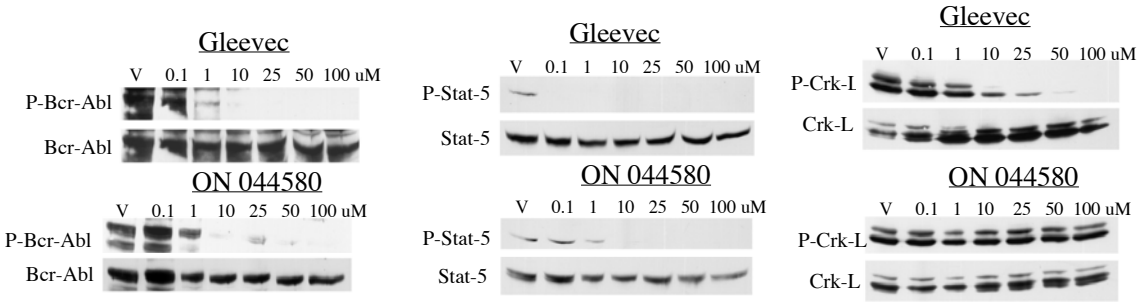

Fig. 2. Inhibition of BCR-ABL Pathway by ON044580. K562 Cells were treated for 2 hours with the indicated concentrations of imatinib or ON044580. Total protein lysates were resolved by SDS-PAGE and subjected to western blot analysis and treated with appropriate antibodies. V= vehicle alone.

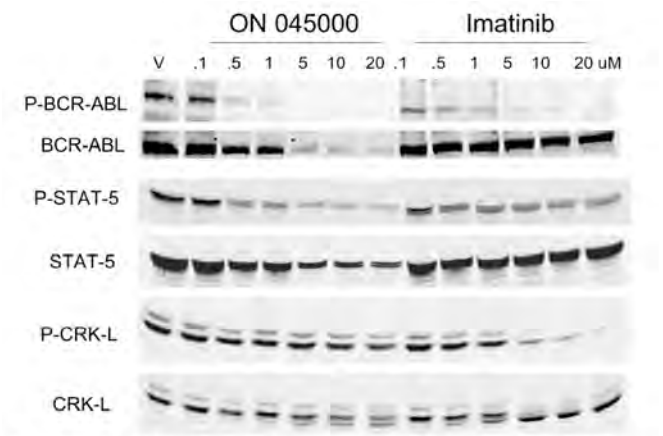

Fig. 3. Inhibition of BCR-ABL Pathway by ON045000. K562 Cells were treated for 2 hours with the indicated concentrations of imatinib or ON044580. Total protein lysates were resolved by SDS-PAGE and subjected to western blot analysis and treated with appropriate antibodies. V= vehicle alone

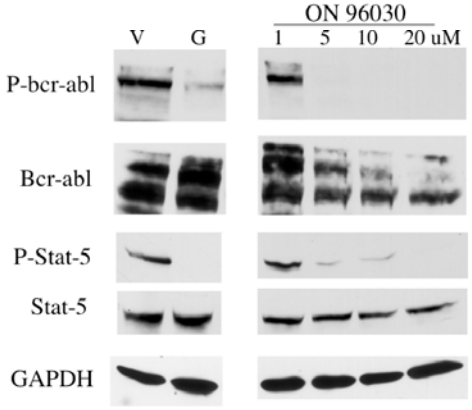

Fig. 4. Inhibition of the BCR-ABL Pathway by ON96030. K562 Cells were treated for 2 hours with 1 uM concentration of imatinib or with the indicated concentrations of ON96030. Total protein lysates were resolved by SDS-PAGE and subjected to western blot analysis with the appropriate antibodies. V= vehicle alone

To evaluate the *in vivo* inhibition of BCR-ABL activity by the compounds under study, we examined the autophosphorylation status of BCR-ABL protein as well as the phosphorylation status of STAT-5 and CrkL in cells treated with these compounds. For these studies, we used K562 cells and 32DCl3 cells expressing the T315I mutant form of theBCR-ABL protein (32D/T315I-BCR-ABL). We treated these cells with increasing concentrations of the three compounds for 2 hrs followed by western blot analysis of cell lysates to determine the ability of these compounds to inhibit the phosphorylation status of BCR-ABL, STAT-5 and CrkL. Data presented in Figures 2,3 and 4 show that all of our compounds inhibit the autophosphorylation of wild type BCR-ABL protein expressed

in K562 cells. Data presented in these figures also shows that all three compounds inhibit the phosphorylation of STAT-5 but not the phosphorylation status of CrkL. Imatinib (Gleevec) was used as a positive control in all these experiments. These results suggest that these compounds are more selective in their inhibitory of activity of BCR-ABL substrates.

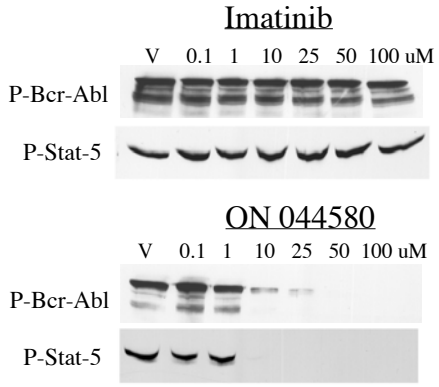

Figure 5: ON044580 inhibits phosphorylation of Bcr-Abl and Stat5 in imatinib-resistant T315I-32D cells. Exponentially growing T315I-32D cells were treated for 2 hours with the indicated concentrations of ON044580. Clarified lysates were subjected to SDS-PAGE followed by Western blotting using the antibodies indicated. V= vehicle alone.

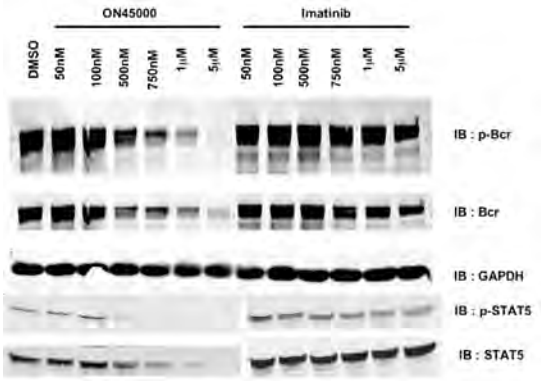

Figure 6: ON045000 inhibits phosphorylation of Bcr-Abl and Stat5 in Gleevec resistant T315I-32D cells. Exponentially growing T315I-32D cells were treated for 2 hours with indicated concentrations of ON045000. Clarified lysates were subjected to SDS-PAGE followed by Western blotting using the antibodies indicated. V= vehicle alone.

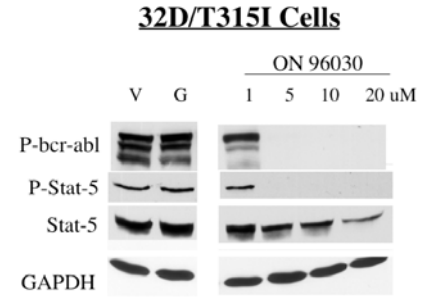

Figure 7: ON96030 inhibits phosphorylation of Bcr-Abl and Stat5 in imatinib-resistant T315I-32D cells. Exponentially growing T315I-32D cells were treated for 2 hours with indicated concentrations of ON96030 or 20uM concentration of imatinib. Clarified lysates were subjected to SDS-PAGE followed by Western blotting using the antibodies indicated. V= vehicle alone..

Following the establishment of their *in vivo* activity towards WT BCR-ABL kinase, we next examined their ability to inhibit autophosphorylation and STAT-5 phosphorylation of T315I-BCR-ABL kinase. For these studies we used the 32D/T315I-BCR-ABL cell line which expresses high levels of the T315I-BCR-ABL kinase and was found to be resistant to imatinib. As was done with K562 cells, we treated 32D/T315I-BCR-ABL cells with increasing concentrations of the three compounds for 2 hrs followed by western blot analysis of cell lysates to determine the ability of these compounds to inhibit the phosphorylation of BCR-ABL and STAT-5. The results of these studies are shown in Figures 5, 6 and 7. These studies show that all three compounds are very effective in inhibiting autophosphorylation and STAT-5 phsophorylation in the 32D/T315I-BCR-ABL cells while imatinib failed to do so. These studies suggest that the three compounds described here do not bind to the ATP-binding

domain of BCR-ABL kinase, but act via binding to the substrate-binding domain (which is specific to STAT-5 but not to Crk-L) or to an allosteric domain of the BCR-ABL kinase that results in the impairment of its ability to phosphorylate itself and STAT-5.

In vitro BCR-ABL kinase inhibitory activity of ON compounds

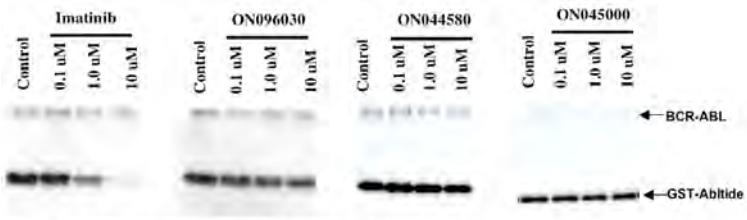

Figure 8: ON096030, ON044580 and ON045000 do not inhibit the *in vitro* kinase activity of recombinant BCR-ABL protein produced in sf9 cells using baculovirus expression vectors. 10ng of recombinant BCR-ABL protein was mixed with different concentrations of the indicated inhibitor and kinase assays were performed using GST-Abltide as a substrate to measure autophosphorylation and substrate phosphorylation.

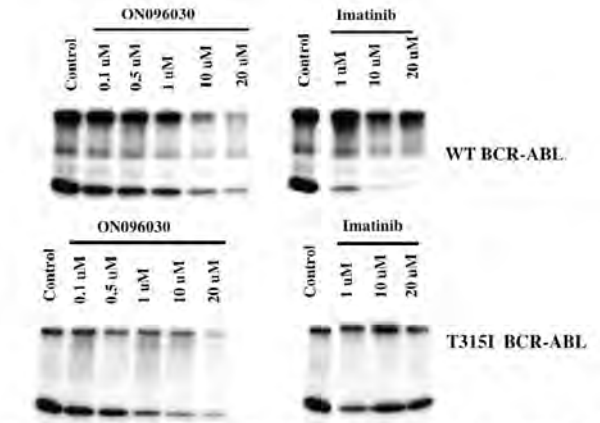

Figure 9. ON096030 inhibits the kinase activity of wild type (WT) and T315I BCR-ABL proteins immunoprecipitated from 32Del3 cells expressing wild-type or T315I BCR-ABL proteins . Cell lysates from 32D/BCR-ABL and imatinib-resistant 32D/BCR-ABL^{T315I} cells were immunoprecipitated with antibodies directed against the BCR-ABL protein. Immunoprecipitates derived from 100ug of the total cell protein was mixed with different concentrations of the indicated inhibitor and kinase assays performed using GST-Abltide as a substrate to measure substrate phosphorylation.

Having determined that the three compounds inhibit wild type (WT) and T315I BCR-ABL activity *in vivo*, we next examined whether these compounds inhibit their *in vitro* kinase activities. This was important since the non-ATP-competitive allosteric inhibitors of BCR-ABL kinase described by Adrian et al (2006) failed to inhibit the *in vitro* kinase activity of recombinant BCR-ABL protein made in insect cells as well as the kinase activity of the protein immunoprecipitated from mammalian cells. For these assays, we first used recombinant full length BCR-ABL protein made in insect cells and examined the effect of the three compounds under study to inhibit the *in vitro* kinase activity using a synthetic peptide, GST-Abltide. Imatinib was used as a positive control. The results of this study (Figure 8) show that all three compounds failed to inhibit the kinase activity of recombinant BCR-ABL kinase while Imatinib could readily do so. We next eamined the ability of these compounds to inhibit the *in vitro* kinase activity of mammalian BCR-ABL proteins

immunoprecipitated from 32D/WT-BCR-ABL or 32D/T315I-BCR-ABL cells. For these studies, cell lysates were prepared from 32D cells expressing either the Wild Type BCR-ABL or T315I mutant form of BCR-ABL and the BCR-ABL protein immunoprecipitated with antibodies directed against the BCR-ABL protein. The immunoprecipitates were washed, re-suspended in kinase buffer and used for kinase assays using the GST-Abltide as a substrate. Immunoprecipitates derived from 100 ug of total protein were used for a single assay reaction and were mixed with different concentrations of the inhibitor. The kinase assys were performed as described previously by us (Gumireddy et al, 2005). Imatinib was used as a control in all of these assays. The results for ON96030 are shown in Figure 9. These studies show that imatinib readily inhibits the kinase activity of WT BCR-ABL but fails to do so with the T315I-BCR-ABL kinase. On the other hand, ON96030 inhibits both WT and T315I mutant forms of BCR-ABL kinase, suggesting that mutations that affect the kinase inhibitory activity of imatinib do not affect the inhibitory activity of ON96030. We carried out similar assays for ON044580 and ON045000 (Figures 10 and 11), and both compounds were effective inhibitors of BCR-ABL kinase activity of the T315I mutant form of BCR-ABL protein.

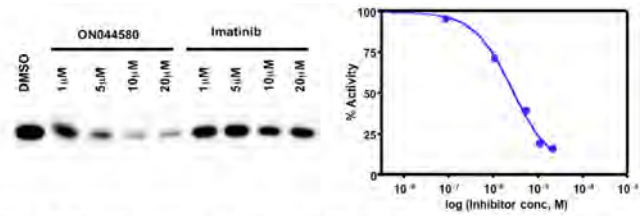

Figure 10. ON044580 inhibits the kinase activity of BCR-ABL^{T315I} protein immunoprecipitated from imatinib-resistant 32D/BCR-ABL^{T315I} cells . Cell lysates from imatinib-resistant 32D/BCR-ABL^{T315I} cells were immunoprecipitated with antibodies directed against the BCR-ABL protein and the immunoprecipitates re-suspended in kinase asssay buffer were used for kinase assays.. Immunoprecipitates derived from 100 ug of total cell protein were used for each assay. Resuspended immunoprecipitates were mixed with different concentrations of the indicated inhibitor and kinase assays performed using GST-Abltide as a substrate to measure substrate phosphorylation.

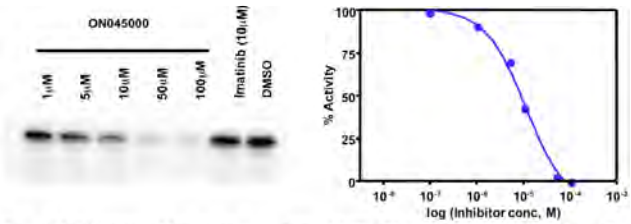

Figure 11. ON045000 inhibits the kinase activity of BCR-ABL^{T315I} protein immunoprecipitated from imatinib-resistant 32D/BCR-ABL^{T315I} cells . Cell lysates from imatinib-resistant 32D/BCR-ABL^{T315I} cells were immunoprecipitated with antibodies directed against the BCR-ABL protein and the immunoprecipitates re-suspended in kinase asssay buffer were used for kinase assays.. Immunoprecipitates derived from100 ug of total cell protein were used for each assay. Resuspended immunoprecipitates were mixed with different concentrations of the indicated inhibitor and kinase assays performed using GST-Abltide as a substrate to measure substrate phosphorylation.

In vitro tumor cell killing activity of ON compounds

We next examined the ability of the three compounds to inhibit the proliferation of BCR-ABL positive myeloid leukemias. For this study, we used K562 cells which express WT BCR-ABL kinase and 32D/T315I-BCR-ABL cells which express an imatinib-resistant

form of BCR-ABL. The results presented in Figure 12 show that all three compounds were effective inducers of myeloid tumor cell death and that ON045000 was the most

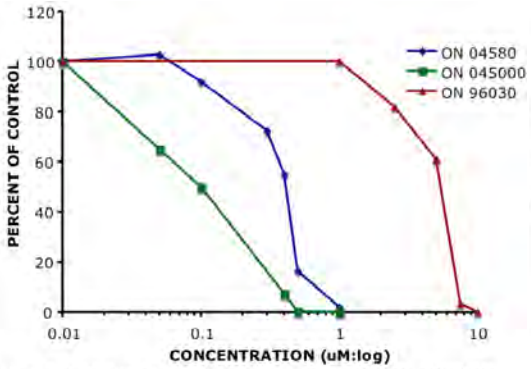

Figure 12. *In vitro* tumor cell killing activity of ON04580, ON045000 and ON96030. K562 cells were plated at 2.5 x 10^4 cells/ml/well. The cells were treated with increasing concentrations of each compound and the total number of viable cells was determined following trypan blue staining and counting using a hemacytometer 96 hours later. The data is plotted as the percent total viable cells compared to DMSO treated controls.

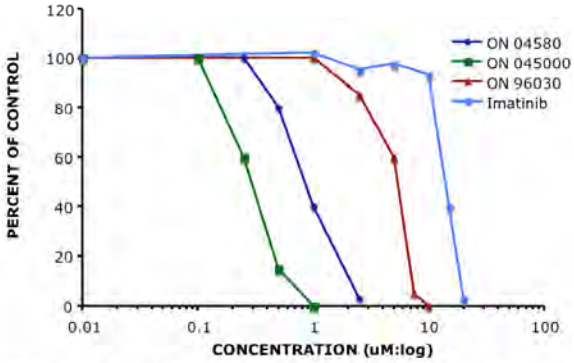

Figure 13. *In vitro* cell killing activity of ON04580, ON045000 and ON96030 using imatinib-resistant 32D/BCR-ABL-T315I cells. 32D/BCR-ABL-T315I cells were plated at 2.5 x 10^3 cells/ml/well. The cells were treated with increasing concentrations of each compound and the total number of viable cells was determined following trypan blue staining and counting using a hemacytometer 96 hours later. The data is plotted as the percent total viable cells compared to DMSO treated controls.

effective with a GI_{50} of 100-200 nM. ON044580 showed a GI_{50} OF 300-400 nM while ON96030 showed a GI_{50} of 3 uM. Imatinib, in the same assay system showed a GI_{50} of 100-200 nM (data not shown).

We next examined the ability of these compounds to inhibit the proliferation of 32D/T315I-BCR-ABL cells. The results presented in Figure 13 show that all three compounds inhibit the proliferation of these cells and that ON045000 was the most active with a GI_{50} of 200 nM. ON044580 showed a GI_{50} of 500-900 nM while ON96030 showed a GI_{50} of 5 uM. Imatinib in the same assay system showed a GI_{50} of 20-30 uM.

JAK2 inhibitory activity of ON compounds

The studies presented above suggest that the three ON compounds inhibit the BCR-ABL kinase either by binding to the STAT-5 binding domain of BCR-ABL or by binding to an allosteric site which results in altered conformation and inhibition of the kinase activity of the protein. It is now well established that STAT-5 is also a substrate of JAK family of kinases, especially JAK2. The JAK family of tyrosine kinase consist of four members, JAK1, JAK2, JAK3 and Tyk2. Most interleukin-mediated activation of hematopoietic cells appears to result predominantly in the activation of JAK-2, although JAK-1 and TYK-2 have also been found to be activated in certain cell systems (Rane and Reddy,

2000). The importance of JAKs in human cancer became evident from recent

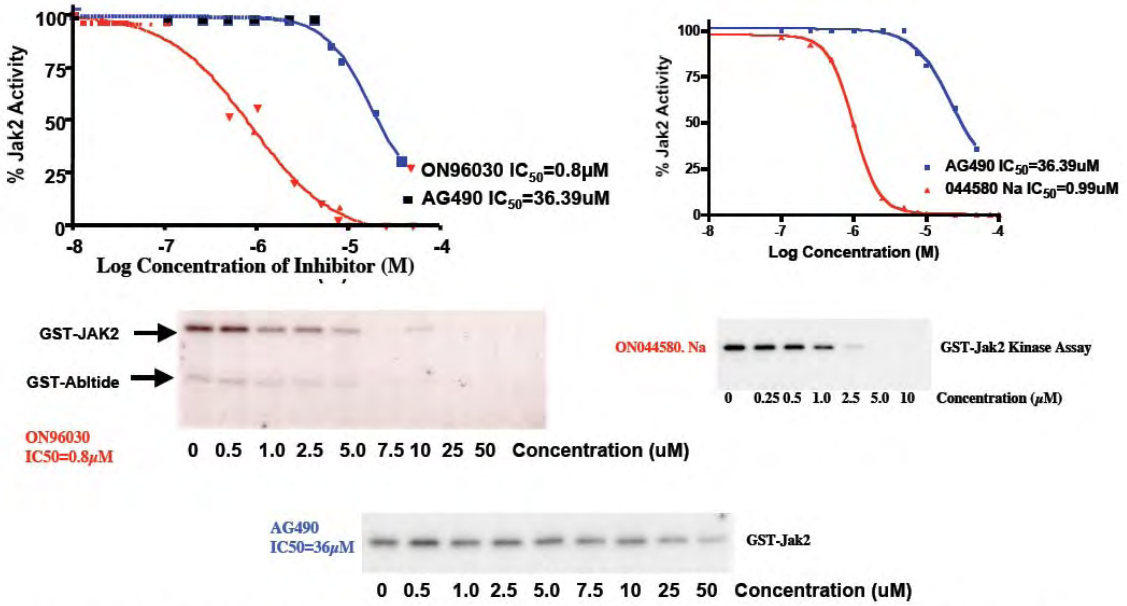

Figure 14. Inhibition of the Jak2 kinase by ONO44580 and ON96030. 10 ng of recombinant JAK-2 kinase was incubated with the indicated inhibitor for 30 minutes at room temperature. The kinase reaciton was initiated by addition of substrate mix (300ng GST-Abltide, 20uM ATP and 10uCi γ32P-ATP) and incubated for 20 mintues at 30°C followed by SDS-PAGE and autoradiography. IC_{50} values were calculated for % inhibition of Jak2 kinase activity (compared to DMSO controls in duplicate) from non-linear regression plots with variable slope using GraphPad Prism4 software.

observations which show that point mutations in the JAK2 kinase (V617F) could be the causative molecular event in certain myeloproliferative disorders (MPDs) (Shannon and Van Etten, 2005). This mutation was found in most patients with polycythemia vera (PV) as well as in some cases of essential thrombocythemia (ET) and chronic idiopathic myelofibrosis (CIMF), all of which are classified as MPDs (Parganas et al, 1998; Baxter et al, 2005; Levine et al, 2005; Meydan et al, 1996). Remarkably, every patient sample contained the same amino acid substitution (V617F) and this mutation has been found to confer erythropoietin-independent growth of the mutant cells *in vitro* (Parganas et al, 1998). As of to-date, the only known inhibitor of JAK-2 is AG-490, which was first described about a decade ago and was found to inhibit JAK-2 with an IC_{50} of 25-50 uM (Meydan et al, 1996). While this compound was found to be an excellent reagent for laboratory investigations, it was found to be unsuitable for clinical development because of its low efficacy and lack of adequate bio-availability. These observations suggest an

urgent need to develop new JAK-2 inhibitors with greater inhibitory activity and enhanced bio-availability. It was therefore of interest to test the three ON compounds for JAK2 inhibitory activity since this will not only provide indirect evidence for substrate-

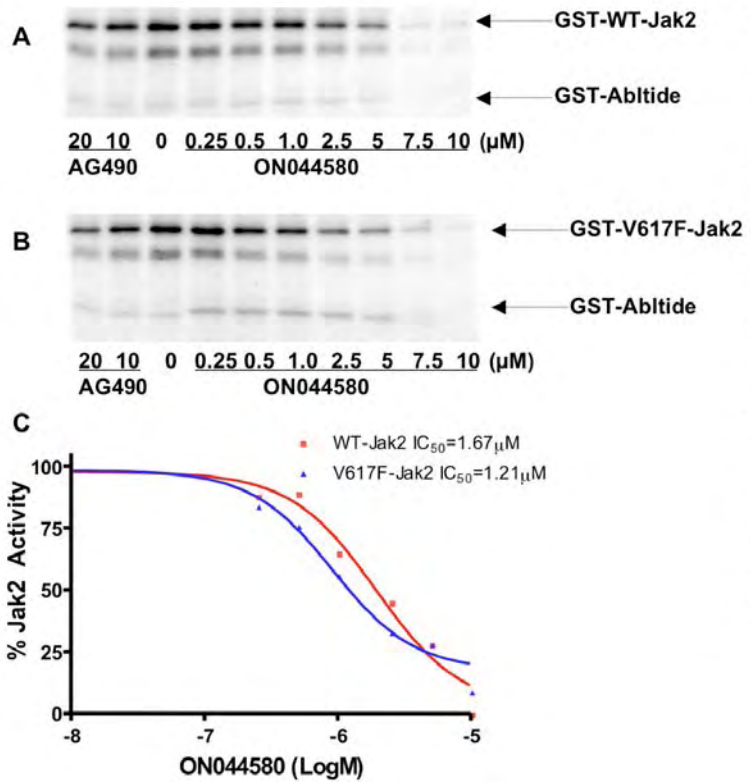

Figure 15. ON044580 inhibits the WT & activated V617F mutant of Jak2. 200 ng of recombinant Wild-type or V617F-Jak2 (aa 532-1124) were mixed with the indicated concentrations of ON044580 at room temperature and radiometric kinase assays performed by addition of 1 µg (1.5µM) of recombinant GST-Abltide, 20 µCi ^{32}P-■-ATP and 20µM unlabelled ATP for 20 minutes at 30ºC. The reaction mixtures were subject to SDS-PAGE and autoradiography. The autoradiograms from Figures 1B and 1C were scanned and the band corresponding to autophosphorylation of Jak2 was quantitated using MacBas software. The densitometric values obtained were plotted as a function of log drug concentration using Prism 4 Graphpad software and IC_{50} values determined by plotting sigmoidal non-linear regression curves with variable slope

competitive nature of our compounds but also could establish their utility for the treatment of MPDs arising due to mutations in JAK2.

To test the JAK2 inhibitory activity of ON compounds, we used both the recombinant protein produced in insect cells (which is commercially available) as well as JAK2 kinase immunoprecipitated from mammalian cells expressing WT and mutant forms of this protein. As of to date, we have completed our studies with ON96030 and ON44580 and the results for these two compounds are presented below. We are in the process of testing the activity of ON045000 against the JAK2 kinase and the results of these studies will be submitted following the completion of these studies. Interestingly, both ON96030 and ON44580 were able to inhibit the kinase activity of recombinant JAK2 in our *in vitro* assays (Figure 14) with an IC_{50} ranging between 0.8 to 1.0 µM. Under identical conditions, AG490 (the only JAK2 inhibitor currently in the market) was able to inhibit

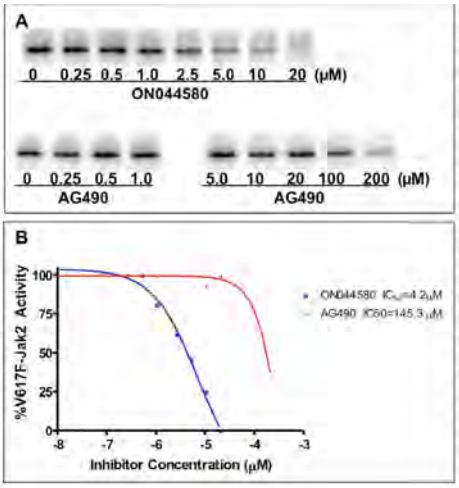

Figure 16: ON044580 Inhibits V617F-Jak2 Immunoprecipitated from IL-3 Stimulated Ba/F3 Cells. **(A)** Jak2 was immunoprecipitated from clarified lysates of BaF3:V617F-Jak2 cells stimulated with 5 ng/ml recombinant IL-3. The washed immunocomplexes were incbuated for 30 minutes in the presence of increasing concentrations of ON044580 and processed for kinase assays. (B) The autoradiograms from A were scanned and the band corresponding to autophosphorylation of Jak2 was quantitated using MacBas software. The densitometric values obtained were plotted as a function of log drug concentration using Prism 4 Graphpad software and IC_{50} values determined by plotting sigmoidal non-linear regression curves with variable slope. While ON044580 showed inhibition with IC_{50}=4.2μM, AG490 had an IC_{50} value of 145.3 μM.

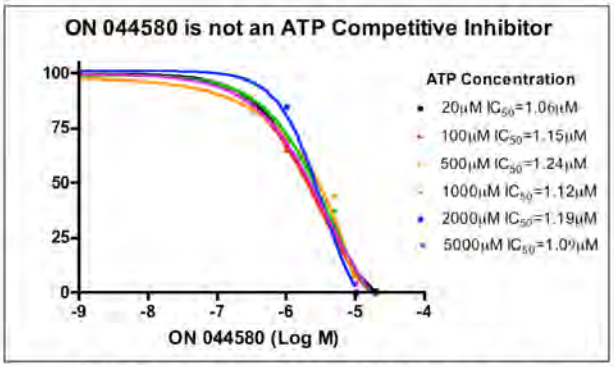

Figure 17: Effect of ATP on V617F-Jak2 inhibitory activity of ON044580. 200 ng of recombinant V617F-Jak2 was mixed with the indicated concentrations of ON044580 and varying concentrations of ATP upto 5 mM. Kinase assays were performed. The values form individual samples were analyzed and plotted as a function of the log of inhibitor concentration.

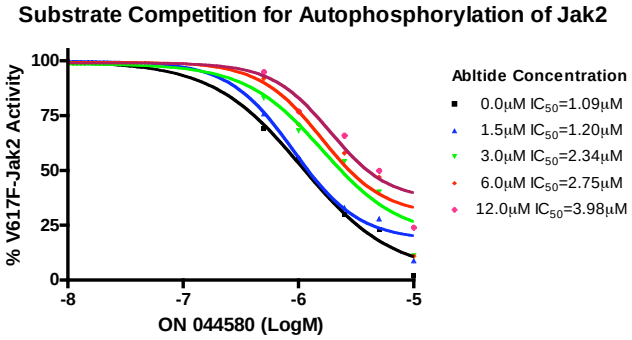

Figure 18: Effect of substrate concentration on the inhibition of V617F-Jak2 autophosphorylation and Abltide phosphorylation. 200 ng of recombinant V617F-Jak2 (aa 532-1124) was mixed with the indicated concentrations of ON044580. Kinase assays performed with varying concentrations of GST-Abltide substrate upto 12.0 μM. The values of individual samples were analyzed and plotted as a function of log inhibitor concentration.

JAK2 kinase activity with an IC_{50} of 36.39 μM, which is in agreement with published literature. Following these observations, we examined the ability of these compounds to inhibit the activated V617F mutant form of JAK2 using commercially available recombinant protein produced in insect cells. The results of this study showed that both compounds inhibit the kinase activity of mutant JAK2 with a similar IC_{50} as that seen with WT JAK2. The results for ON044580 are shown in Figure 15. Because the recombinat preparations of WT and mutant JAK2 proteins are truncated forms of the kinase, we examined the kinase inhibitory activity of these compounds using JAK2 kinase immunoprecipitated from the Baf3:V617F-JAK2 cell line which expresses the full length form of mutant JAK2 (Provided to us by Dr. Ralph Arlinghaus). These studies again showed that the two compounds inhibited the JAK2 kinase activity with an IC_{50} of approximately 3-4 μM. The results for ON044580 are shown in Figure 16. Under identical conditions, AG490 inhibited the kinase activity with an IC_{50} of 145 μM (Fig. 16).

ON compounds are non-ATP competitive inhibitors. The observation that ON compounds inhibit recombinant JAK2 allowed us to test whether these compounds bind to the ATP binding domain or the substrate-binding domain of JAK2. For this, we carried out kinase inhibition assays either in the presence of increasing amounts of ATP or in the presence of increasing amounts of substrate. The results of this study shown in Figures 17, demonstrate that increasing the ATP concentration in the kinase reaction mixture did not affect the inhibitory activity of ON044580. On the other hand, increasing the substrate concentration in the reaction mixture resulted in an inhibition of the kinase inhibitory activity of ON044580 (Figure 18).

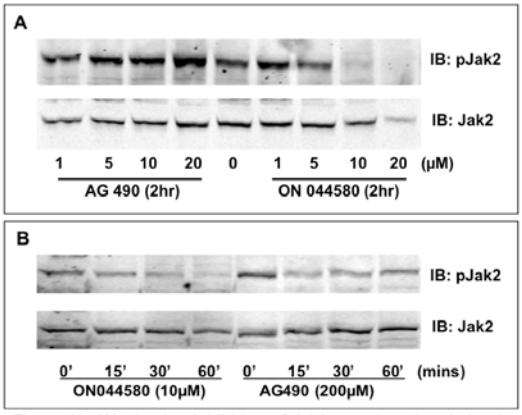

Figure 19: (A). In vivo inhibition of Jak2 autophosphorylation by ON044580 in Ba/F3:V617F-Jak2 cells. Mid-log phase cells growing in the presence of recombinant IL-3 were treated for 2 hours with indicated concentrations of ON044580. Washed cells were lysed in detergent containing buffer and the clarified lysates subjected to SDS-PAGE followed by Western blotting. **(B). ON044580 inhibits Jak2 autophosphorylation in Ba/F3:V617F-Jak2 cells within 15 minutes.** Mid-log phase cells growing in the presence of IL-3 were treated for the indicated times with 10 μM ON044580 or 200 μM AG490 and processed as above.

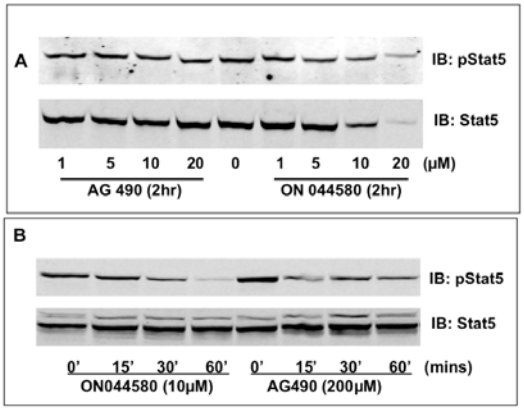

Figure 20: In vivo inhibition of Stat-5 phosphorylation by ON044580 in Ba/F3:V617F-Jak2 cells. (A) Mid-log phase cells were treated for 2 hours with indicated concentrations of ON044580 or AG490. Washed cells were lysed in detergent containing buffer and the clarified lysates subjected to SDS-PAGE followed by Western blotting. **(B) ON044580 inhibits Stat-5 phosphorylation in Ba/F3:V617F-Jak2 cells within 30 minutes.** Mid-log phase cells growing in the presence of IL-3 were treated for the indicated times with 10 μM ON044580 or 200 μM AG490 and processed as above.

***In vivo* inhibition of Jak2 autophosphorylation and STAT-5 phosphorylation by ON044580 in Ba/F3:V617F-Jak2 cells.** To test the *in vivo* kinase inhibitory activity of ON044580, we treated Ba/F3:V617F-Jak2 cells with increasing concentrations of the compound for 2 hrs in the presence of recombinant IL3 (which enhances the phosphorylation status of JAK2). At the end of the 2 hr incubation period, cells were washed and lysed in detergent containing buffer and the clarified lysates subjected to SDS-PAGE followed by western blotting to detect the phosphorylation status of JAK2. The results of this study, presented in Figure 19A, showed that ON044580 was able to inhibit the phosphorylation of JAK2 in a concentration dependent manner. AG490, under

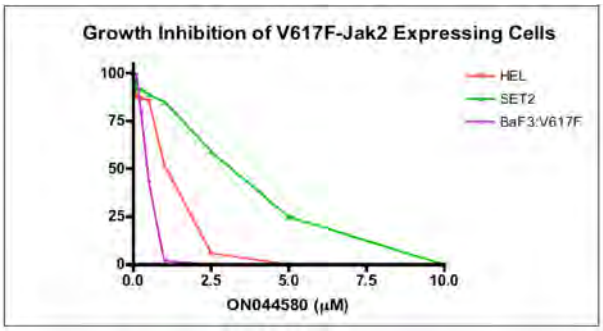

Figure 21: Growth inhibition of V617F-Jak2 expressing cells. Ba/F3:V617F-Jak2 cells, HEL cells (homozygous V617F-Jak2) and SET-2 cells (hemizygous V617F-Jak2) were grown in the presence of varying concentrations of ON044580 for 72 hours. Cell viability was measured by trypan blue exclusion. Data is plotted as the precent total viable cells compared to DMSO controls. HEL is a human cell line derived from a erythroleukemia patient. SET2 is a human cell line derived from a essential thrombocythemia patient. In a screen for the V617F-Jak2 mutation in leukemic cell lines, HEL cells were found to be homozygous while SET-2 cells have been described as hemizygous for the mutation.

identical conditions did not inhibit JAK2 phosphorylation, which could be due to the low IC_{50} values seen for full length JAK2 kinase with this compound (please see Figure 16). As part of this study, we also examined the time course of inhibition where we added 10 μM of ON044580 for periods of time ranging from 15 to 60 minutes and examined the phosphorylation status of JAK2 using western blot analysis. The results of this study presented in Figure 19B showed that in as little as 15-30 minutes, the compound was able to inhibit JAK2 phosphorylation.

Using a similar approach, we also examined the phosphorylation status of STAT-5 (a natural substrate of JAK2) in Ba/F3:V617F-Jak2 cells treated with increasing concentrations of the compound. The results presented in Figure 20 show that ON044580 inhibits STAT-5 phosphorylation in a concentration-dependent and time-dependent manner. Since similar results were seen with JAK2 phosphorylation, these studies suggest that the two events are interrelated.

Growth inhibition of V617F-Jak2 expressing cells. To determine whether ON044580 inhibits the proliferation of V617F-Jak2-positive leukemic cells, we studied its effect on the growth and viability of three different cell lines which express the mutant form of Jak2. These included the Ba/F3:V617F-Jak2 cells which were transfected with an expression vector that encodes the mutant Jak2 and two human leukemic cell lines that were derived from leukemic patients that naturally contained this mutation in their Jak2 loci. One of them, HEL, was homozygous for V617F mutation while the second cell line, SET2 was hemizygous for the V617F mutation. The results of this study, presented in Figure 21 show that ON044580 could readily inhibit the proliferation and induce apoptosis of all the three cell lines at nM or low uM concentrations. Thus the GI_{50} for

Ba/F3:V617F-Jak2 cells was approximately 250 nM while the GI_{50} for HEL cells was approximately 600 nM. Interestingly, the SET2 cell line which was hemizygous for V617F mutation was more resistant to the cell killing activity of the compound with a GI_{50} value of 3.0 μM. We are currently investigating the molecular mechanisms associated with this variability.

Key Research Accomplishments.

1. We have developed three novel small molecule inhibitors of BCR-ABL that inhibit the proliferation and induce apoptosis of CML cell lines that express the WT or the T315I mutant form of BCR-ABL.

2. The three ON compounds were found to be non-ATP competitive and readily induced the down-regulation of BCR-ABL auto-phosphorylation and STAT-5 phosphorylation.

3. The ON compounds described here were also found to inhibit kinase activities of WT and V617F mutant forms of JAK2.

4. The ON compounds described here were also found to inhibit JAK2 and STAT-5 phosphorylation in vivo in cell lines that express V617F form of JAK2.

5. These compounds were also found to inhibit the proliferation and induce apoptosis of leukemic cell lines that express the V617F mutant form of JAK2 and establish their utility for the treatment of MPDs arising due to mutations in JAK2.

Reportable Outcomes

None

Conclusions

1. Non-ATP competitive inhibitors of BCR-ABL are effective inhibitors of imatinib-resistant forms of BCR-ABL including the T315I-BCR-ABL.

2. Because of their substrate-competitive nature, some of these inhibitors also inhibit JAK2 kinase activity.

3. These compounds are useful therapeutic agents for CML as well as MPDs arising due to mutations in JAK2

References

Adrian, F.J., Ding, Q., Sim, T., Velentza, A., Sloan, C. Liu, Y., et al. 2006. Allosteric inhibitors of Bcr-abl-dependent cell proliferation. Nature Chem. Biol. 2, 95-102.

Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S et al. 2005. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet. 365(9464):1054-61.

Deininger, M., Buchdunger, E. and Druker, B.J. 2005. The development of imatinib as a therapeutic agent for chronic myeloid leukemia. Blood 105, 2640-2653.

Gumireddy, K., Baker, S.J., Cosenza, S.C., John, P., Kang, A.D., Robell, K.A., Reddy, M.V., Reddy, E.P. 2005. A non-ATP-competitive inhibitor of BCR-ABL overrides imatinib resistance. Proc Natl Acad Sci U S A. 102(6):1992-1997.

Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJ, et al. 2005. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell. 7(4):387-97.

Meydan N, Grunberger T, Dadi H, Shahar M, Arpaia E, Lapidot Z, et al. 1996. Inhibition of acute lymphoblastic leukaemia by a Jak-2 inhibitor. Nature. 15;379(6566):645-8.

O'Hare T, Walters DK, Deininger MW, Druker BJ. 2005. AMN107: tightening the grip of imatinib. Cancer Cell. 7, 117-119.

Parganas E, Wang D, Stravopodis D, Topham DJ, Marine JC, Teglund S, et al. 1998. Jak2 is essential for signaling through a variety of cytokine receptors. Cell. 93(3):385-95.

Work Accomplished by Dr. Arlinghaus' Group at the M.D. Anderson Cancer Center

INTRODUCTION:

Because the Arlinghaus lab has shown that the Jak2 tyrosine kinase is a critical downstream target of the Bcr-Abl oncoprotein (Samanta et al, 2006), we have investigated the effects of ON 044580 on the signaling pathways driven by Bcr-Abl in BCR-ABL+ leukemia cells. Our initial results indicate that ON 044580 causes relatively rapid induction of apoptosis in Bcr-Abl+ cells compared to AG490, in both imatinib-sensitive and resistant cells including the T315I mutant. The T315I Bcr-Abl mutant is the most difficult IM-resistant mutant to control (Shah et al, 2004). Therefore, we wanted to explore the mechanism, which is responsible for this rapid apoptosis induction. Because a known HSP90 inhibitor (17-AAG) also caused rapid induction of apoptosis in Bcr-Abl+ cells and because HSP90 would be expected to maintain the structural matrix of the Bcr-Abl/Jak2 protein Network (see Samanta et al, 2006), we examined the effects of ON 044580 on HSP90 expression. Our findings indicate that ON 044580 caused a reduction of HSP90 expression, and a reduction in its client proteins. This reduction of HSP90 appears to lead to a disruption of a protein network involved in Bcr-Abl signaling. We hypothesize that reduction of HSP90 releases the client proteins from the HSP90

chaperone complex, which leads to a rapid induction of apoptosis. These client proteins include Bcr-Abl, Akt, and possibly other members of the Bcr-Abl/Jak2 signaling Network. We note that Stat3 and MAPK are thought to regulate expression of HSP90 expression (Chatterjee et al, 2007). These client proteins, as a result of being released from their chaperone, are known to be degraded by way of the proteasome pathway, which would lead to apoptosis of the leukemia cell. In Bcr-Abl+ cells this effect seems to result from the simultaneous inhibition of both Bcr-Abl and Jak2 tyrosine kinases. Blocking the Jak2 kinase pathway only also leads to apoptosis but at a slower rate (e.g. 72 hr) compared to more rapid apoptotic induction (e.g. 36 hr) resulting from simultaneous Bcr-Abl and Jak2 tyrosine kinase inhibition. Studies with "normal cells" are planned. Such experiments would include parental cell lines (e.g. BaF3/32D mouse cells maintained in IL-3), and mouse and human peripheral blood mononuclear cells (PBMCs) in culture and normal marrow cells from mice.

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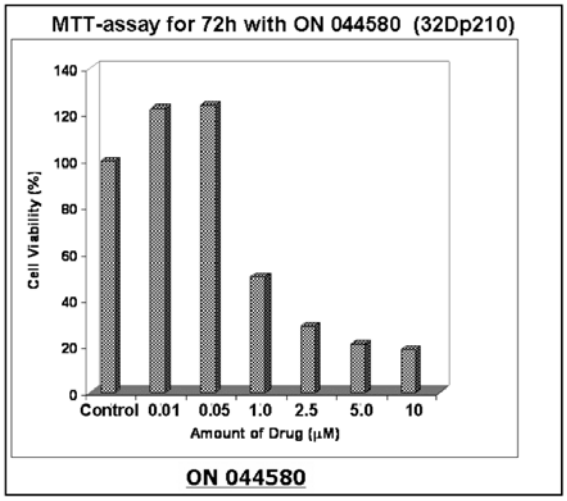

Fig. 1. Inhibitory effects of ON 044580 on th e viability of mouse hematopoietic cells transduced with the Bcr-Abl oncoprotein, termed P210 BCR -ABL. These experiments were performed using the MTT assay over a 72 hr time period.

ON 044580 reduces viability of Bcr-Abl+ cells. We tested the effects of ON 044580 on the viability of Bcr-Abl+ 32D cells using the MTT assay. These cells were derived from an IL-3 dependent mouse myeloid cell clone that requires IL-3 for growth and survival. Expression of Bcr-Abl in these cells abrogates the requirement for IL-3 to maintain survival. Viability as measured by MTT assays indicates that 1 µM level of ON 044580 severely inhibits viability of these cells (IC50=1 µM) (Fig. 1). Imatinib (IM) resistance has several forms. One important form of IM resistance involves activation of the Lyn kinase to maintain the

oncogenicity of the CML cell despite treatment with IM (Donato et al, 2003., Donato et al, 2004). IM does not inhibit the kinase activity of Lyn, a Src tyrosine kinase family member expressed in hematopoietic cells. Treatment of this cell line with ON 044580, which is resistant to effects of high levels of IM, strongly reduced viability at concentration of 10 μM, with an IC50 of about 2.5 μM (Fig. 2). The inhibition was observed in a dose-dependent manner. Similarly, the viability of IM-resistant mutants, including the gatekeeper mutant (T315I) were strongly inhibited by the drug (Fig. 3).

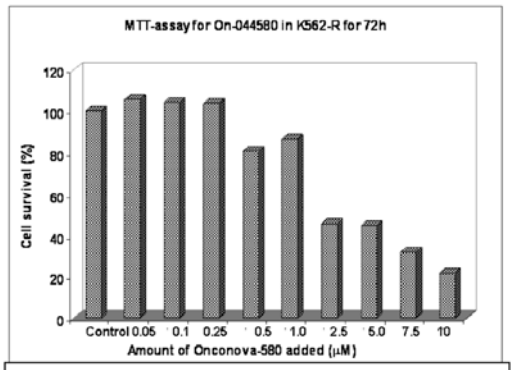

Fig. 2. Inhibitory effects of ON 044580 on the viability of CML cell line K562-R, which is resistant to imatinib mesylate (IM). K562-R cells are resistant to IM because they over-express the active Lyn tyrosine kinase. IM does not inhibit Lyn kinase. Therefore, K562 R cells survive and cycle even though Bcr-Abl is inhibited. The MTT assay was performed as in Fig. 1.

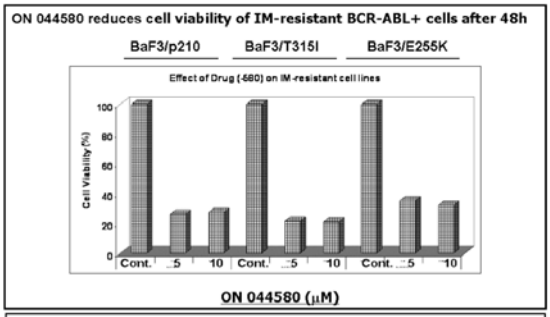

Fig. 3. ON 044580 severely reduces the viability of IM-sensitive and resistant mouse cells expressing BCR-ABL. Viability was measured by the Trypan blue dye exclusion method. It is very significant that the gatekeeper IM-resistant mutant is strongly inhi bited by ON 044850.

ON 044580 induces apoptosis in cells expressing Bcr-Abl, both IM-sensitive and IM-resistant cells. We assessed the ability of ON 044580 to induce apoptosis in several different hematopoietic cells lines expressing Bcr-Abl (Fig. 4-10). The oncogenic behavior of cells expressing Bcr-Abl is in part due to their ability to be resistant to apoptosis induction. Apoptosis was assessed by the annexin V/PI method using flow cytometry. Expression of annexin V on the cell surface is an early indication of apoptosis. Propidium iodide uptake by cells is a manifestation of a late stage apoptotic cell. Our studies show that ON 044580 is a potent inducer of apoptosis at concentrations of 10 μM or below. IC50s range from 1 to 5 μM. Of interest, the K562-R cell line, which is very resistant to apoptosis caused by IM, is very sensitive to ON 044580 (IC50 of about 2.5 μM) (Fig. 5). Similarly, hematopoietic cells expressing the T315I IM-resistant mutant of BCR-ABL were also quite responsive to apoptotic induction by ON 044580, having an IC50 below 2.5 μM (Fig. 8). The T315I mutant is termed the gatekeeper mutation, since all known kinase inhibitors that target the ATP binding

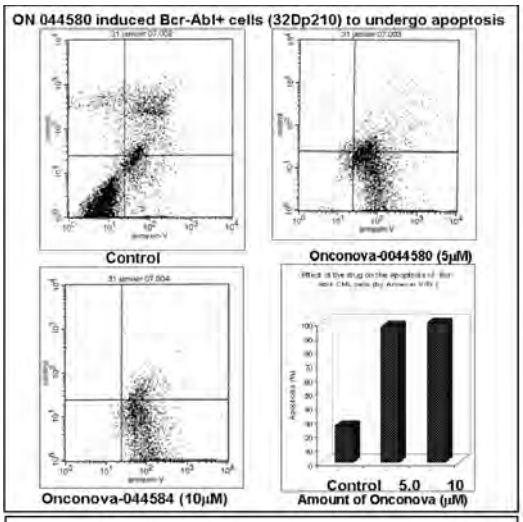

Fig. 4. ON 044580 induces apoptosis in Bcr-Abl+ 32D cells in a dose-dependent manner. Mouse 32D cells (myeloid blastic cells) transformed by Bcr-Abl induce leukemia in mouse models whereas 32D cells do not. Apoptosis was measured after 72 hr of treatment with ON 044850 using the annexin 5/PI method involving flow cytometry. Apoptotic cells accumulate in the lower right (quadrant #3) and upper right quadrants (#4). The bar graph is the total number of cells in quandrants 3 and 4.

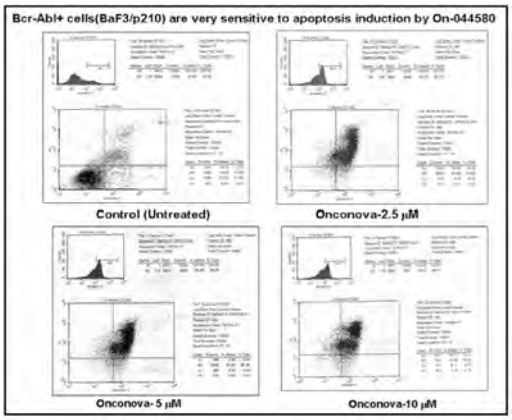

Fig. 6. ON 044580 induces apoptosis in mouse lymphoid cells (BaF3) transformed by Bcr-Abl. As in Fig. 4, ON 044580 induces apoptosis in BaF3 cells (lymphoid lineage) transformed by Bcr-Abl. Apoptosis was measured by annexin V/PI as in Fig. 4 and 5.

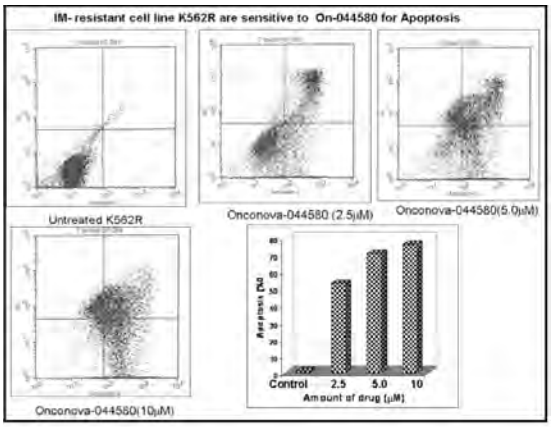

Fig. 5. ON 044580 indu ces apoptosis in IM-resistant CML cell line K562-R in a dose-dependent manner. Apoptosis was measured by the annexin V/PI method as in Fig. 4. These cells are imatinib (IM) resistant because they contain an activated Lyn tyrosine kinase, which drives the leukemic phenotype despite the inhibition of Bcr-Abl by IM. Thus, ON 044580 induces cell killing in CML cells that do not depend on Bcr-Abl.

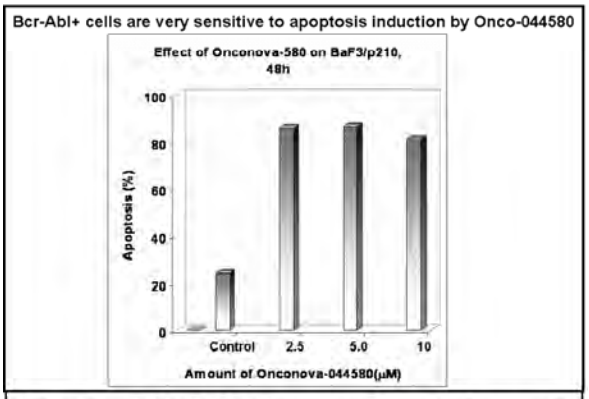

Fig. 7. ON 044580 is a pot ent inducer of apoptosis in BCR-ABL+ BaF3 cells. The results shown in Fig. 6 were quantitated by flow cytometry.

domain of the Bcr-Abl tyrosine kinase fail to induce apoptosis in T315I cells (Shah et al, 2004). Therefore, it is quite significant that ON 044580 is able to induce apoptosis in T315I mutant cells. Similar results were obtained with the E255K IM-resistant mutant of Bcr-Abl (Fig. 9, 10).

ON 044580 induces apoptosis in primary cells from CML patients. At this point in our studies, we tested cells from three patients with CML. One is a blast crisis CML patient, which is refractory to IM treatment (Fig. 11). The other is a CML patient in blast crisis. Both responded to induction of apoptosis by ON 044580 (Fig. 11 and 12). A third cell

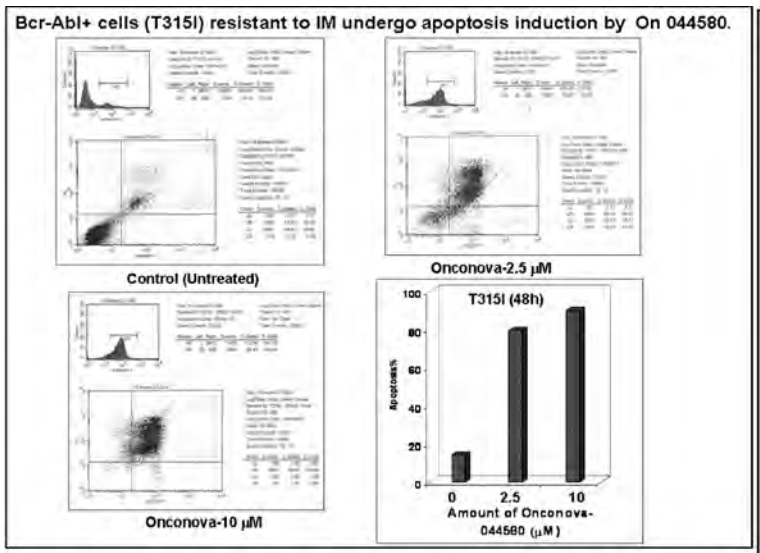

Fig. 8. ON 044580 induces apoptosis in BaF3 cells transformed by th e IM-resistant Bcr-Abl mutant (T315I); these cells are resistant to imatinib, dasatinib and nilotinib tyrosine kinase inhibitors. Apoptosis was measured by annexin 5/PI flow cytometry method. The T315I mut ation is a so called gatekeeper mutant because this mutation prevents effective binding of all the known tyrosine kinase inhibitors like imatinib, which block A TP binding to the kinase. ON-044580, being an inhibitor of the catalytic domain of Abl, effectively blocks the Bcr-Abl tyrosine kinase effects.

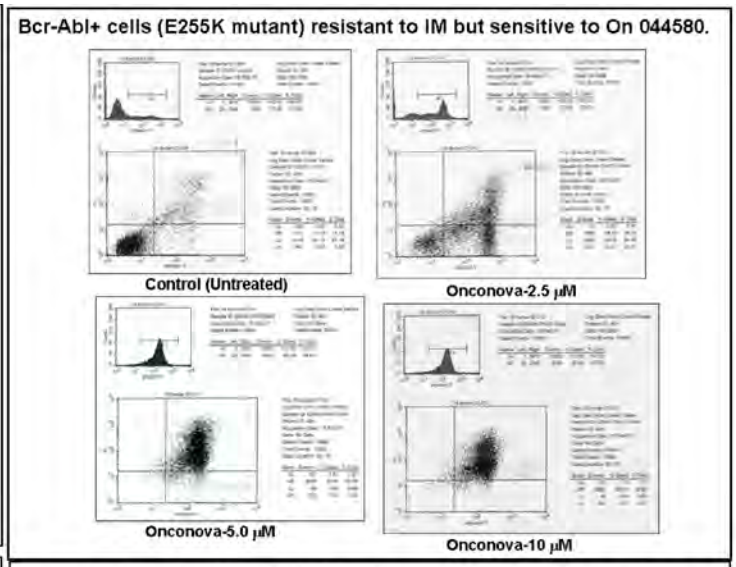

Fig. 9. Fig. 9. ON 044580 in duces apoptosis in BaF3 cells transformed by the IM-resistant E255K mutant of Bcr-Abl. Annexin 5/PI flow cytometry method was used to assess apoptosis activity of ON 044850. Unlike the T315I mutation, the E255K IM-resistant mutant form of Bcr-Abl is sensitive to dasatinib and nilotinib. ON 044580 also induces apoptosis in cells transformed by the E255K mutant of Bcr-Abl, suggesting that all IM-resistant forms of Bcr-Abl will be effectively inhibited by ON 044850.

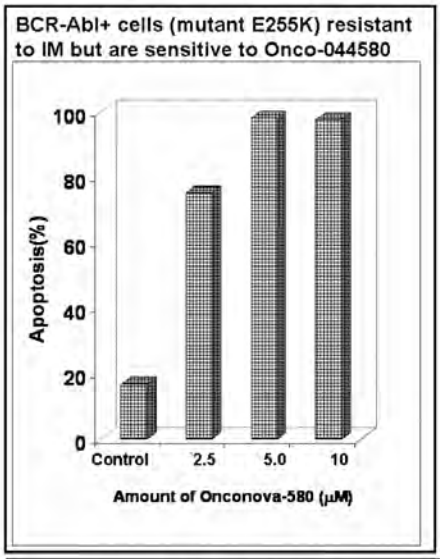

Fig. 10. ON 044580 is very effective at blocking the an ti-apoptotic activity of the E255K IM-resistant mutant of B cr-Abl.. This figure shows the qua ntitative inhibitory effects of ON 044580 on cells transformed by the E255K IM-resistant mutant of Bcr-Abl.

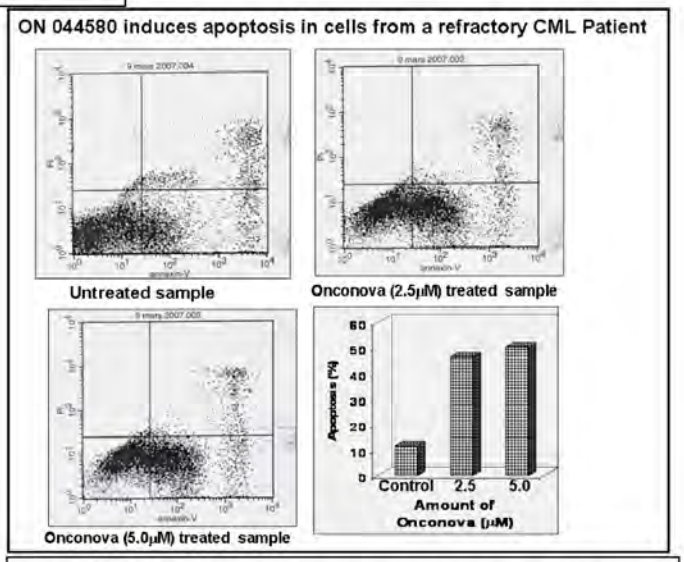

Fig. 11. ON 044580 ind uces apoptosis in cells from refractory CML patient. A blood sample was received through our approved Lab protocol. The patient was a blast crisis CML patient refractory to IM treatment. White blood cells were maintained in c ulture with medium containing cytokine growth factors for 58 hr in the presence of various doses of ON 044580. Cells we re assayed for apoptosis by the annexinV/PI method.

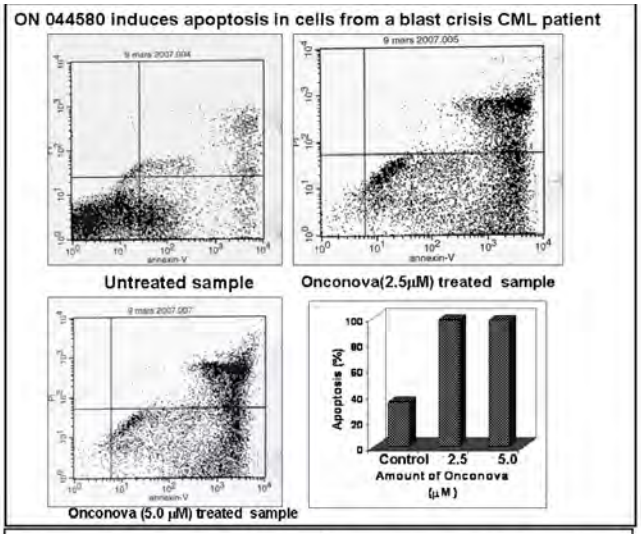

Fig. 12. ON 044580 induces apoptosis in cells from a b last crisis CML patient. Cells were processed as in Fig. 11. This patient was a blast crisis stage CML patient.

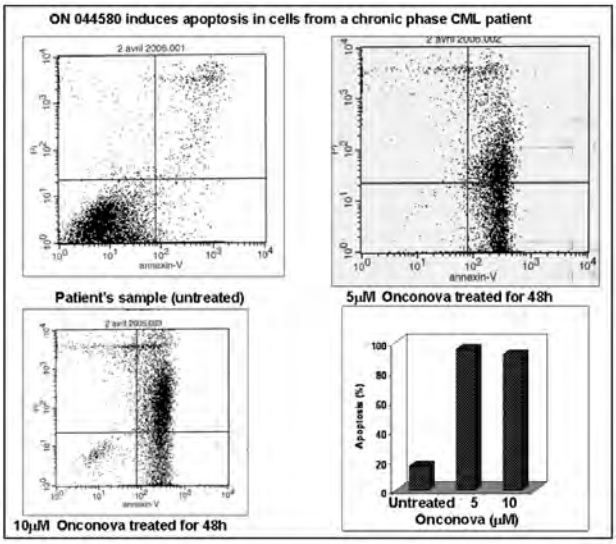

Fig. 13. ON 044580 induces apoptosis in cells from a chronic phase CML patient.

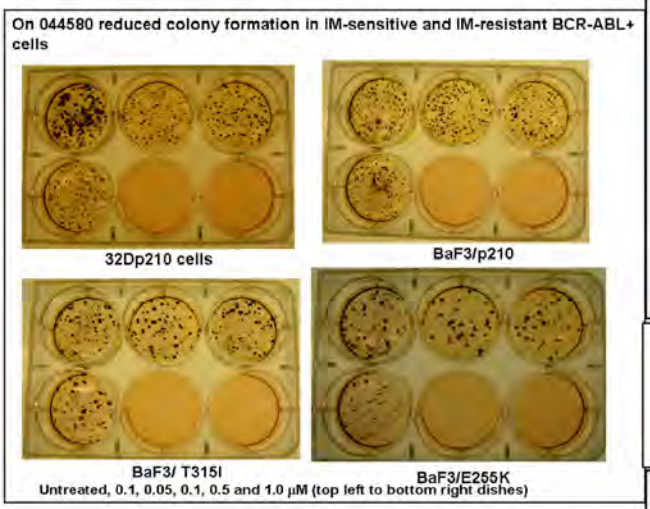

Fig. 14. ON 044580 inhibits anchorage-independent growth of Bcr-Abl+ mouse hematopoietic cells both sensitive and r esistant to IM. Cells were seeded in soft agar dishes such that one cell must form a colony, and grown for two weeks. Colonies were stained for counting and photography. Anchorage-independent growth is an indicator of the cells ability to form tumors in animals

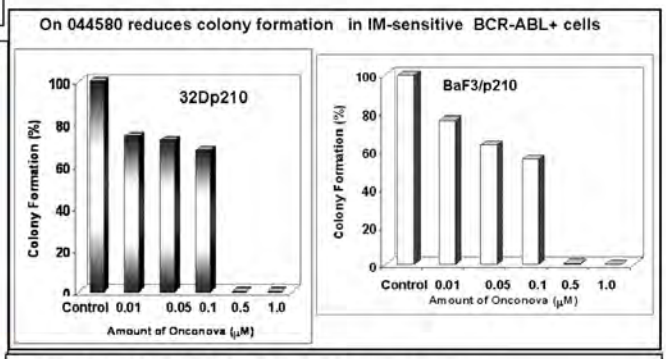

Fig. 15. ON 044580 inhibits anchorage-independent growth of BCR-ABL+ cells-Quantitative assessment. Colonies were stained and counted.

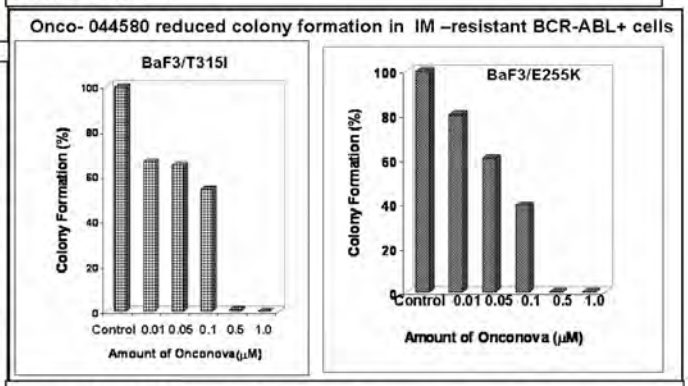

Fig. 16. ON 044580 inhibits anchorage-independent growth of IM-resistant (T315I and E255K) BCR-ABL+ cells-Quantitative assessment.

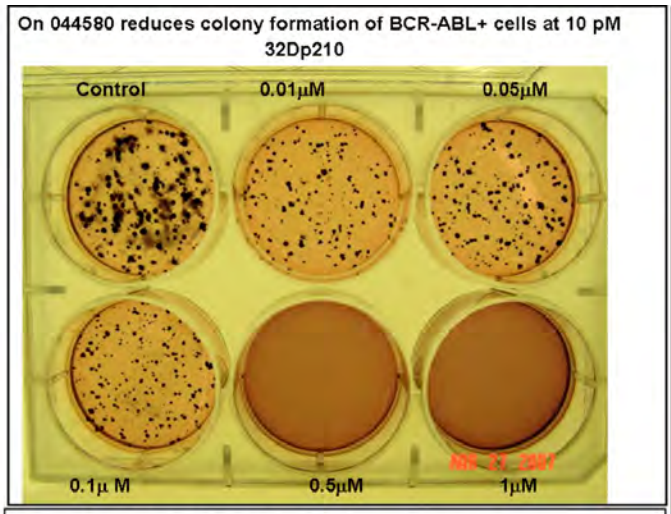

Fig. 17. ON 044580 inhibits anchorage-independent growth of mouse BCR-ABL+ 32D cells at sub μM concentrations (10 nM).

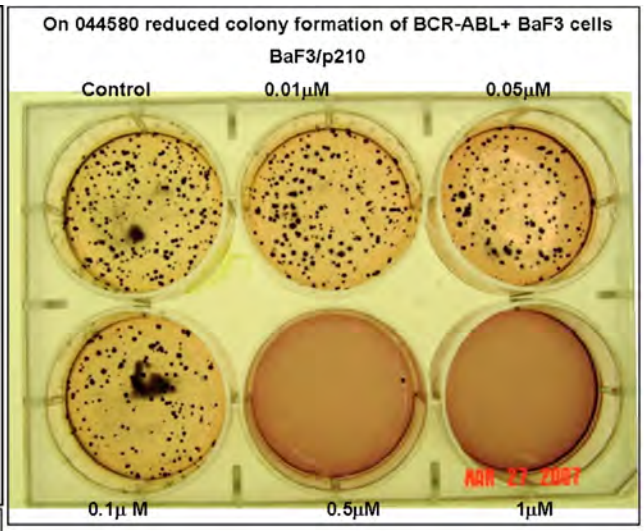

Fig. 18. ON 044580 inhibits anchorage-independent growth of mouse BCR-ABL+ BaF3 cells at sub uM concentrations (below 1 uM).

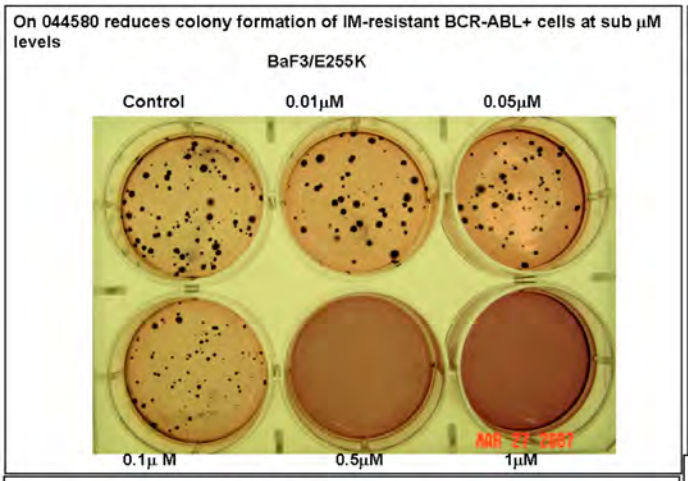

Fig. 19. ON 044580 inhibits ancho rage-independent growth of IM-resistant (T315I) mouse BCR-ABL+ BaF3 cells at sub uM concentrations (below 1 uM).

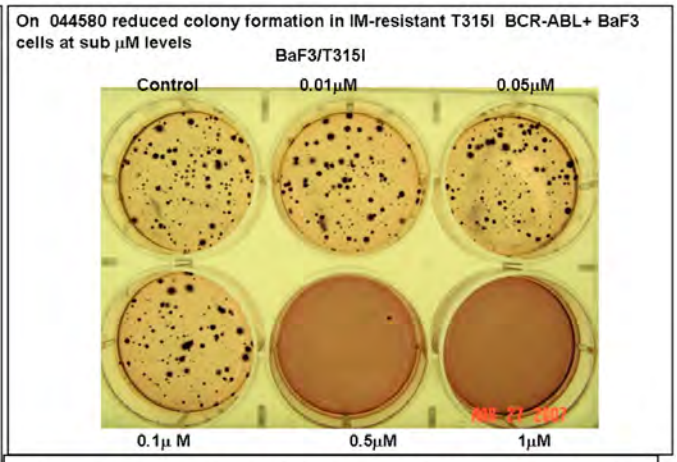

Fig. 20. ON 044580 i nhibits anchorage-independent growth of IM-resistant (E255K) mouse BCR-ABL+ BaF3 cells at sub uM concentrations (below 1 μM).

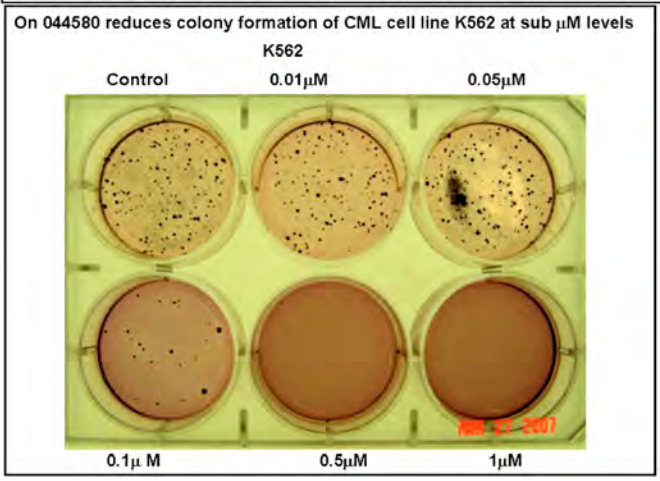

Fig. 21. ON 044580 in hibits anchorage-independent growth of K562 CML cells at sub uM concentrations (below 1 μM).

sample is from a CML patient in chronic phase (Fig. 13). Note that the blast crisis and chronic phase cells were highly sensitive to apoptosis induction by ON 044580, which suggests that ON 044580 may be useful to treat the unusually resistant blast crisis patients. Cells were maintained for 48 hr in the absence of cytokines, to enhance the level of Bcr-Abl+ cells in the blood cell population. Cells were treated for an additional 48 hr with various doses of the drug.

ON 044580 inhibits anchorage-dependent growth of Bcr-Abl cells that are both sensitive and resistant to apoptosis. Anchorage-independent growth is a cell culture surrogate indicator for tumor behavior in animals. We assessed the effects of ON 044580 on colony formation in soft agar cultures. Cells were seeded into soft agar culture medium at the single cell level. Cultures were allowed to incubate for two weeks. Colonies were stained, photographed and counted to quantitate the effects of the drug. Cells that were both IM-sensitive and IM-resistant were tested (Fig. 14-21). In general, colony formation was completely inhibited at 0.5 μ M ON 044580. Importantly, IM-resistant forms of Bcr-Abl were also inhibited at similar concentrations (Fig. 14, 16). Because the sharp drop in colony forming ability at levels at or above 0.5 μM, the effects of ON 044580 were-tested at 0.01 to 1 μM. (Fig. 17-21). The results showed that ON 044580 severely inhibited colony formation at levels between 0.1 and 0.5 μM. Of interest, BaF3 cells grown in IL-3 also formed colonies, and ON 044580 had no detectable affect on colony formation up to 0.25 μM ON 044580 (not shown). Some experiments showed that colony formation by IM-resistant mutant Bcr-Abl BaF3 cells were reduced at levels below 0.1 μM (Fig. 19-20). Further studies on planned on this aspect of the project.

ON 044580 inhibits tyrosine phosphorylation of IM-sensitive and resistant Bcr-Abl+ cells.

We tested the effects of ON 044580 on the pattern of tyrosine-phosphorylated proteins in Bcr-Abl+ cells, both sensitive and resistant to IM. (Fig. 22). Not only were tyrosine-phosphorylated proteins strongly decreased by ON 044580, but interestingly the level of Bcr-Abl oncoprotein was also severely reduced, even to the point that the Bcr-Abl protein was undetectable after treatment with 10 μM of the drug (Fig. 22, top right). Top left and

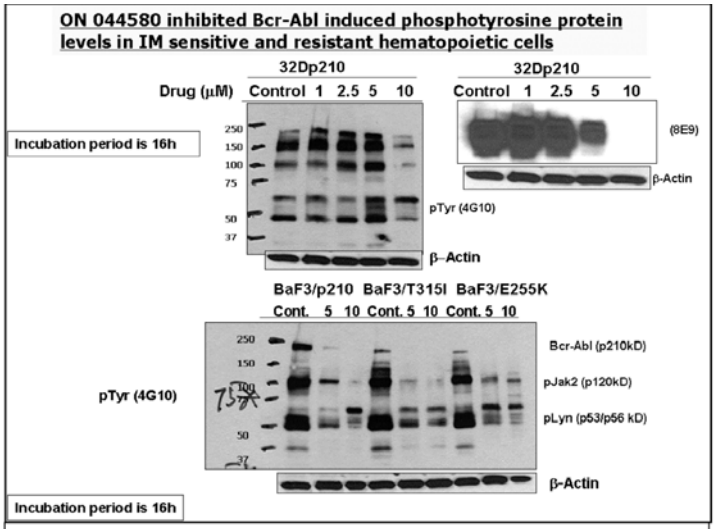

Fig. 22. ON 044580 reduces the level of phosphotyrosine proteins in IM-sensitive and resistant BCR-ABL+ hematopoietic cells. Cells were lysed in in a detergent-based buffer containing high levels of protease and phosphatase inhibitors. The proteins were analyzed by Western blotting using 4G10 anti-phosphotyrosine antibody. Of interest, 0N 044850 severely reduced the level of the Bcr-Abl oncoprotein, suggesting that the inhibition of the Bcr-Abl kinase also decreases the level of the oncoprotein as a secondary effect. As we shown below, simultaneous inhibition of Jak2 and Bcr-Abl tyrosine kinases leads to the reduction of HSP90 chaperone protein, which we propose reduces the stability of a variety of proteins in the Bcr-Abl/Jak2 Network.

bottom were blotted with antibody to phosphotyrosine (4G10). The top right pattern was developed with anti-Abl (8E9). Anti-8E9 is a mouse monoclonal antibody that detects the Bcr-Abl and c-Abl proteins with a very high degree of specificity (Guo et al, 1994). Equal amount of protein was loaded onto each gel as indicated by the anti-actin blot.

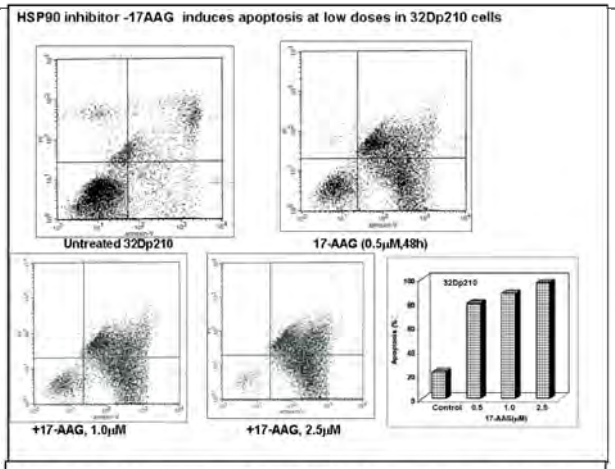

Fig. 23. Inhibition of HSP90 chaperone protein by the known HSP90 inhibitor 17 AAG in duces apoptosis in BCR-ABL+ cells.

<u>The HSP90 inhibitor 17AAG induces apoptosis in 32D cells expressing Bcr-Abl.</u> Heat shock protein 90 (HSP90) plays a key role in conformational maturation of oncogenic signaling proteins, including HER2/neu, Akt, Raf-1, Bcr-Abl and p53 (Kamal et al, 2004). It has been shown that tumor cells have a 100-fold higher binding affinity for 17AAG than does HSP90 from normal cells (Kamal et al, 2004). Because we have shown that Bcr-Abl and Jak2 exist in a large protein network (Samanta et al, 2006), we anticipate that HSP90 would be involved in maintaining the structure of the Bcr-Abl/Jak2 protein Network. Figure 23 shows that 17AAG was a potent inducer of apoptosis, with an IC50 of less than 0.5 μM.

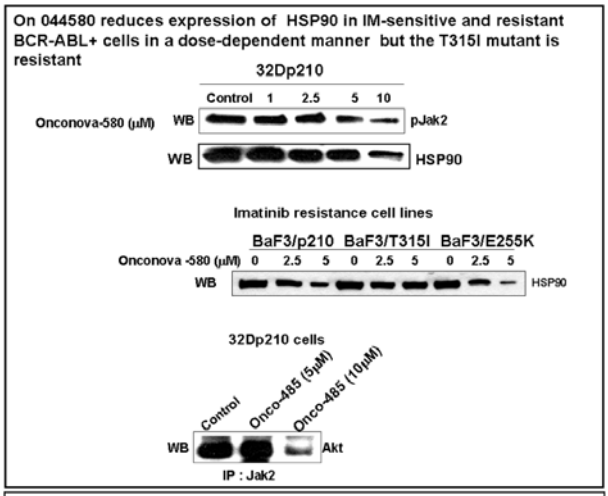

Fig. 24. ON 044580 reduces the level of HSP90 protein expression in a dose-dependent manner in Bcr-Abl+ IM- sensitive and resistant cells (E255K) but T315 Bcr-Abl+ cells are resistant to the reduction of HSP90.

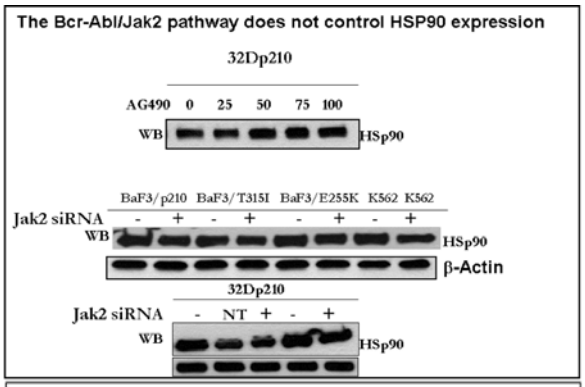

Fig. 25. Inhibition of the Jak2 tyrosine kinase alone does not inhibit expression of HSP90 in Bcr-Abl+ cells. AG490 treatment (Jak2 inhibition) or knockdown of Jak2 expression by Jak2 siRNA did not reduce the expression of HSP 90., suggesting that simultaneous inhibition of both the Jak2 and Bcr-Abl pathways is required for reduction of HSP90 expression. These results suggest that 0N 044580 inhibits other targets besides Jak2 and Bcr-Abl.

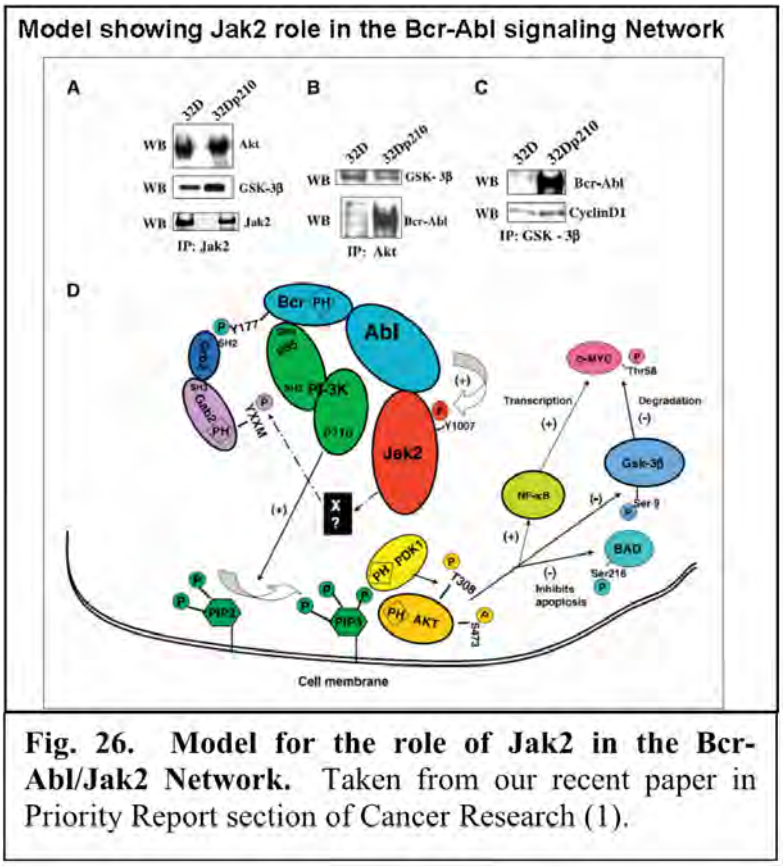

Fig. 26. Model for the role of Jak2 in the Bcr-Abl/Jak2 Network. Taken from our recent paper in Priority Report section of Cancer Research (1).

ON 044580 reduces the level of HSP90 in IM-sensitive and resistant cells Bcr-Abl+ cells.

Reduction of HSP90 in Bcr-Abl+ cells by ON 044580 was seen in a dose dependent manner (Fig. 24). Of interest, IM-resistant cells expressing the T315I mutant Bcr-Abl protein appear to be resistant to HSP90 down-regulation by ON 044580 (Fig. 24). However, these same cultures undergo apoptosis by drug treatment (Fig. 8), and showed a severe reduction in the colony forming ability (Fig. 16,

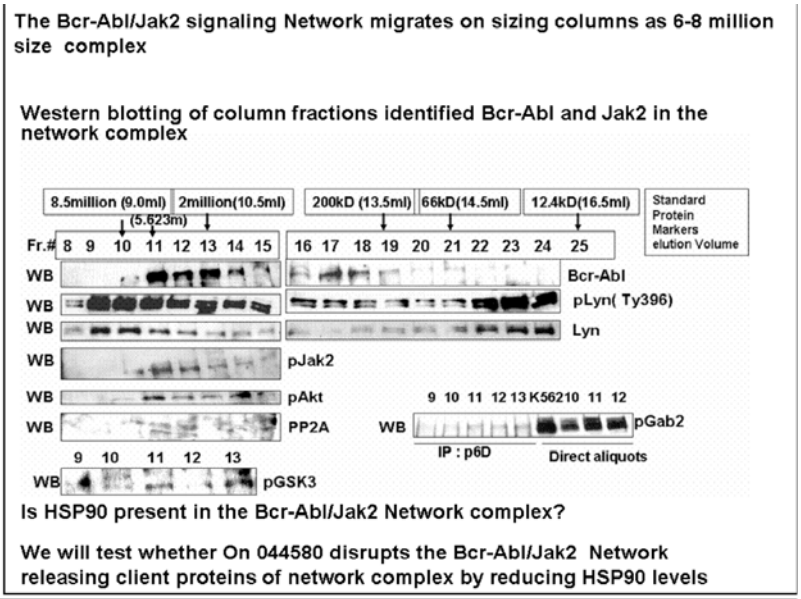

Fig. 27. The Bcr-Abl Network exists in a 6-8 million size complex as judged by fractionation of a gel filtration column chromatography. The CML cell line K562 was lysed in our detergent buffer containing high levels of protease and phosphatase inhibitors. The clarified lysates was applied to a gel filtration column, which allows separation of complexes of up to 8 million in size. Various standard molecular weight markers were resolved on this column, allowing positions of proteins from 68 kDa up to 6-8 million daltons. Fractions were analyzed by Western blotting with very antibodies to proteins that we have detected in co-immunoprecipitation experiments in Bcr-Abl+ cells (1). Note that Bcr-Abl eluted in two regions; one estimated to be about 6-8 million Daltons and the other about 200,000 daltons

19) and a severe reduction in viability (Fig. 3). Thus, ON 044580 is effective for killing T315I Bcr-Abl+ cells but the mechanism does not appear to involve reduction of HSP90. We used a compound known to reduce HSP90 as a control (17AAG, ref) (Fig. 23). Of importance, the Bcr-Abl/Jak2 pathway did not appear to down-regulate HSP90, as neither the Jak2 inhibitor AG490 nor knockdown of Jak2 reduced levels of HSP90 in cells from IM-sensitive and resistant Bcr-Abl+ cells (Fig. 25). Models for the effects of ON 044580 are shown for BCR-ABL+ cells in Fig. 28 and 29.

The Bcr-Abl/Jak2 Network elutes on a gel filtration column with an estimated molecular size 6-8 million molecular size. To estimate the relative size of the Bcr-Abl/Jak2 Network, we fractionated a lysates of K562 cells on a gel filtration column, which was eluted with a buffer containing NP-40. Fractions were analyzed by Western blotting with various antibodies to several proteins thought to be present in this Network (Fig. 27). Bcr-Abl, pTyr (Y1007) Jak2 pSer Akt, pTyr (YxxM) Gab2, pSer GSK3 beta co-eluted in the 6-8 million size fractions. Bcr-Abl also eluted in the 200 kDa fractions. Further

studies are planned to determine whether HSP90 is present in the Bcr-Abl/Jak2 Network and what the effect of ON 044580 is on this structure. If HSP90 functions as expected, this structure will collapse upon treatment with sufficient concentration of ON 044580 (e.g 1-10 μM).

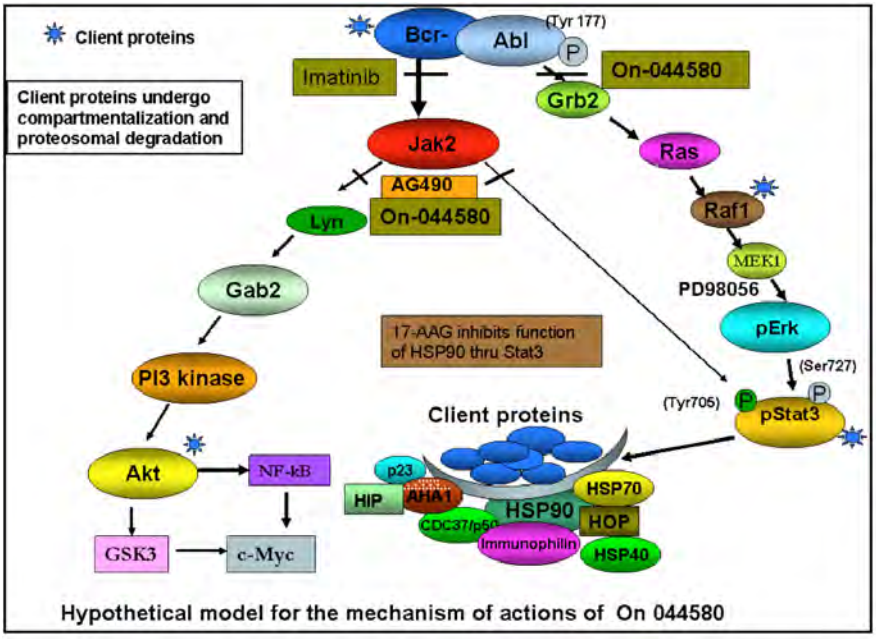

Fig. 28. Model for effects of 0N 044580 in Bcr-Abl+ cells. Studies by Dr. Reddy's group indicate that ON 044580 inhibits both the Bcr-Abl and Jak2 tyrosine kinases. The blue star identifies client proteins for the HSP90 chaperone protein.

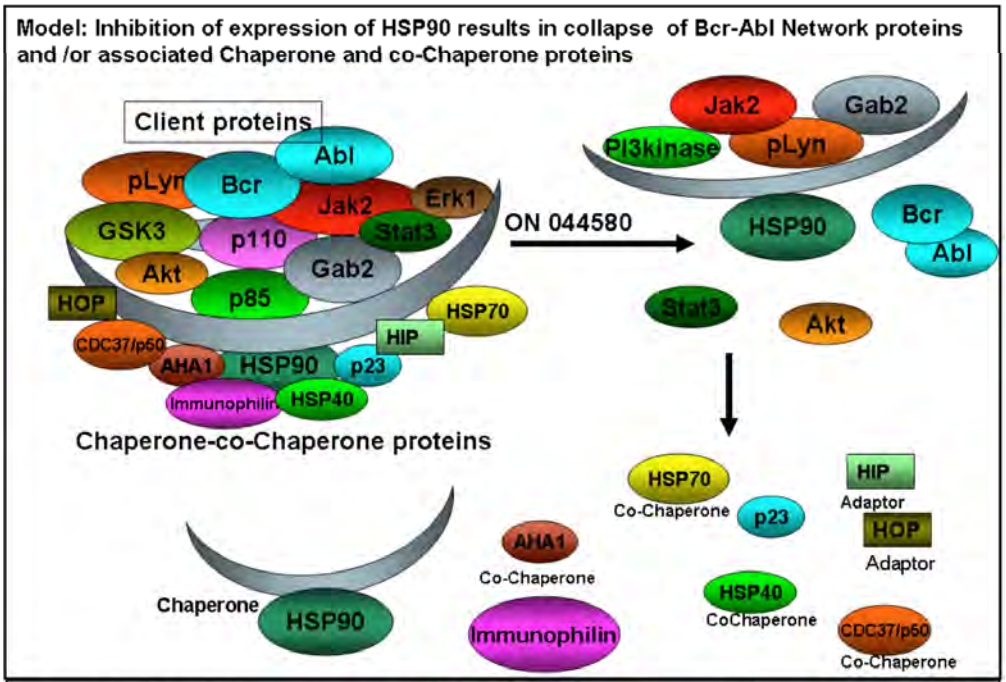

Fig. 29. Model for effects of ON 044580 on the stability of the HSP90 chaperone structures. Briefly, ON 044580 by reducing expression of HSP90 is proposed to cause a collapse of the Bcr-Abl/Jak2 Network complex.

KEY RESEARCH ACCOMPLISHMENTS:

1. ON 044580 induces rapid apoptosis in Bcr-Abl+ cells (either cell lines and primary patient cells), both sensitive and resistant to imatinib mesylate compared to either imatinib mesylate or AG490
2. ON 044580 strongly inhibits anchorage-independent growth of Bcr-Abl+ cells, both sensitive and resistant to imatinib mesylate
3. ON 044580 reduces expression of HSP90, a chaperone protein likely to be involved in maintaining the structure of the Bcr-Abl/Jak2 Network (see ref. 1).
4. A compound (17AGG) known to reduce expression of HSP90, induces rapid apoptosis in Bcr-Abl+ cells, both sensitive and resistant to imatinib mesylate. These findings support the hypothesis that ON 044580 induces rapid apoptosis in Bcr-Abl+ cells by disrupting the HSP90-bound Bcr-Abl/Jak2 client proteins in the Bcr-Abl/Jak2 Network.

REPORTABLE OUTCOMES:

None at this time.

CONCLUSIONS:

Imatinib mesylate (Gleevec, Novartis Pharmaceutical) is a very successful medicine, which is widely used for treatment of Philadelphia chromosome positive chronic myeloid leukemia (CML). ON 044580 induces extensive apoptosis in cells expressing and transformed by both imatinib-sensitive and resistant mutants of Bcr-Abl. The most resistant mutant form is the T315I mutant form, which has been shown to be resistant to all of the newer drugs that have the same mode of action as imatinib, which include dasatinib (Bristol Myers Squibb) and nilotinib (Novartis). Thus, ON 044580 has potential for therapy of these most resistant forms seen in imatinib treated CML patients.

Of importance, ON 044580 appears to act through reduction of the chaperone HSP90 in Bcr-Abl+ cells. HSP90 is likely to be required for the structural integrity of the Bcr-Abl/Jak2 Network, which we have described recently (Samanta et al, 2206). This effect along with its ability to inhibit the catalytic domains of Jak2 and Abl tyrosine kinase bodes well for its ability to control patients with all or most forms of imatinib resistance. Of interest is the ability of ON 044580 to induce apoptosis in another important form of imatinib resistance, which involves over-expression of Lyn kinase (Donato et al, 2003, 2004).

Future plans: Because of the unexpected findings that HSP90 appears to be involved in the Bcr-Abl/Jak2 protein Network, we will investigate the mechanism of HSP90 up-regulation by Bcr-Abl, and the mechanism by which ON 044580 down-regulates HSP90 expression, which leads to rapid apoptosis, and the signaling pathway involved.

REFERENCES:

Chatterjee, Jain, M., Stuhmer, S., Andrulis, T., Ungethum, M., Kuban, U., Lorentz, R.J., Bommert, H., Topp, K., Kramer, M., Muller-Hermelink, D., Einsele, H.K., Greiner, H., Bargou, A. R. C. STAT3 and MAPK signaling maintain overexpression of heat shock proteins 90alpha and beta in multiple myeloma cells, which critically contribute to tumor-cell survival. Blood, 15 January, 109 (2), 2007.

Donato, N. J., Wu, J. Y., Stapley, J., Gallick, G., Lin, H., Arlinghaus, R., and Talpaz, M. BCR-ABL independence and LYN kinase over-expression in chronic myelogenous leukemia cells selected for resistance to STI571. Blood, *101:* 690-698, 2003.

Donato, N. J., Wu, J. Y., Stapley, J., Lin, H., Arlinghaus, R., Aggarwal, B. B., Shishodia, S., Albitar, M., Hayes, K., Kantarjian, H., and Talpaz, M. Imatinib mesylate resistance through BCR-ABL independence in chronic myelogenous leukemia. Cancer Res, *64:* 672-677, 2004.

Guo, J. Q., Lian, J. Y., Xian, Y. M., Lee, M. S., Deisseroth, A. B., Stass, S. A., Champlin, R. E., Talpaz, M., Wang, J. Y., and Arlinghaus, R. B. BCR-ABL protein expression in peripheral blood cells of chronic myelogenous leukemia patients undergoing therapy. Blood, *83:* 3629-3637, 1994.

Kamal, A., Thao, L., Sensintaffar, J., Zhang, L., Boehm, M. F., Fritz, L. C., and Burrows, F. J. A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. Nature, *425:* 407-410, 2003.

Kamal, A., Boehm, M. F., and Burrows, F. J. Therapeutic and diagnostic implications of Hsp90 activation. Trends Mol Med, *10:* 283-290, 2004.

Samanta, A.K, Lin, H., Sun, T., Kantarjian, H., and Arlinghaus, R.B. Jak2: A critical target in chronic myelogenous leukemia. Cancer Research (Priority report) 66: (13); July 1. 2006.

Shah, N.P., Tran, C., Lee, F.Y., Chen, P., Norris, D., and Sawyers, C.L. (2004). Overriding imatinib resistance with a novel ABL kinase inhibitor. Science *305*, 399-401.

Work Accomplished by Onconova Therapeutics

Body

1. Onconova chemists synthesized approximately 3000 compounds to screen for BCR-ABL inhibitors by Dr. Reddy's group.

2. Onconova scientists worked in collaboration with Dr. Reddy's group to develop the high thru put screening methos described in Dr. Reddy's report.

3. Onconova developed methods for large scale synthesis of ON043580, ON045000 and ON96030 and supplied gram quantities of these compounds to Drs. Reddy and Arlinghaus.

4. Onconova scientists have initiated studies to determine the oral bio-availability of ON043580 and ON045000 in rodent and dog model systems.

Key Research Accomplishments

Please see Dr. Reddy's and Dr. Arlinghaus group's reports.

Reportable Outcomes

None

Conclusions

We have developed methods for the large scale production of ON043580, ON045000 and ON96030 for pre-clinical and clinical development of these compounds.